

**ESTROGENS, GENETIC POLYMORPHISMS AND BREAST CANCER RISK IN  
NIGERIAN WOMEN**

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CANCER RISK IN NIGERIAN WOMEN**

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University of Pittsburgh, 2005

Breast cancer is major cause of morbidity and mortality globally and the incidence appears to be rising faster in population groups that hitherto experience lower incidence. This case control study recruiting 250 women with breast cancer and 250 age-matched controls from four University Teaching Hospitals in Nigeria was designed to evaluate the risk factors for breast cancer in Nigerian women. Family history of breast cancer was associated with a 15-fold increased risk of breast cancer [Odd ratio (OR) = 14.99, 95% Confidence interval (CI), 1.98, 113.47]. Also, waist to hip ratio (OR = 2.10, 95% CI 1.44, 3.06), history of abortion (OR = 2.83, 95% CI 1.12, 7.19), increasing age at first childbirth (OR = 1.39 95% CI 1.11, 1.73) and higher level of education (OR = 1.31, 95% CI 1.07, 1.61) conferred increased risk of breast cancer. Increasing parity (OR = 0.87, 95% CI 0.77, 0.99) and increasing duration of breastfeeding (OR = 0.75, 95% CI 0.62, 0.91) conferred protection against breast cancer. In the final multivariate conditional logistic regression in all women, carrying at least one low-activity COMT (Met) allele was associated with a significant 43% reduced risk of breast cancer (OR = 0.57, 95% CI 0.36-0.91). While harboring the CYP1A1 M1 polymorphic variant was associated with non-significant reduced risk of breast cancer (OR = 0.79, 95% CI 0.48-1.29), the CYP1A1 M3 polymorphism conferred a non-significant 24% reduced risk of breast cancer (OR = 0.76, 95% CI 0.47-1.22). Results of this study have important public health implications; it has provided evidence for a role for reproductive and other variables in susceptibility to breast cancer in

indigenous African women, thus contributing to the global epidemiologic literature on risk factors for breast cancer in populations of African ancestry. It has also provided data suggesting protection for breast cancer for women harboring the low-activity COMT (Met) allele of the codon 158 polymorphism of the COMT gene. In addition, the findings of this study will serve a useful resource tool in future research and policy decisions aimed at breast cancer control and prevention in these populations.

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Michael N. Okobia

## **1. INTRODUCTION**

Breast cancer is the most common form of cancer among women, and the leading cause of death related death among women globally (Parkin et al, 1999). The lifetime risk of developing breast cancer is reportedly 1 in 8 for women in North America, and 1 in 12 for Western Europe. The incidence of breast cancer in women has been rising since the 1940s and the rise is occurring more rapidly in population groups that hitherto enjoyed a low incidence of the disease (Parkin et al, 1999). For many years, breast cancer incidence and mortality rates have been highest in North America and Northern Europe, intermediate in Southern Europe and Latin America, and lowest in Asia and Africa (Parkin et al., 1997). The steep rise in incidence in most countries of Northern Europe and North America from the early 1980s has been ascribed to the introduction of mammographic screening for breast cancer (Kelsey et al. 1993). In recent years, steep increases in breast cancer incidence and mortality rates have been reported in many Asian and Central European countries. Thus the magnitudes of the differences in incidence rates between countries such as Japan and the United States are less than they were previously (Kelsey et al., 1993). Over time, the world breast cancer burden has increased steadily with an almost doubling of the annual number of estimated new cases over a 20-year span, the increase being seen both in the developed as well as the developing countries (Parkin et al., 1988); Parkin et al., 1985; Parkin et al., 1999; Sasco AJ, 2001).

International differences in breast cancer incidence rates has been hypothesized to be partially related to variation in such factors as body weight (De Waard et al 1977), some aspects of diet (Amstrong et al., 1975), hormone levels (Henderson et al., 1991), and reproductive

characteristics especially age at menarche (Henderson et al., 1991), and possibly menstrual cycle length, parity, and lactation (Wang et al., 1992). Differences in hormone levels among women in various countries have also been thought to play a role (Bernstein et al., 1993). Studies of migrants to the United States suggest that environmental factors rather than genetic factors are mainly responsible for the variation in breast cancer incidence rates among countries.

### **Incidence of Breast Cancer in US Populations**

There are racial/ethnic variations in the incidence and mortality rates of breast cancer among the various ethnic/racial groups in the United States. Breast cancer is the most common cancer among women in every major ethnic group in the United States. Estimates of age-adjusted incidence rates per 100,000 by race/ethnicity in California for the 1988-1989 were 110.6 for white women, 96.3 for black women, 59.2 for Hispanic women, and 52.8 for Asian American women (Hoegh et al., 1992). Incidence rates are higher in whites than blacks above the age of 40-45 years, but below this age range, blacks are at slightly higher risk than whites (Hoegh et al., 1992). Younger average age at first birth among blacks appears to be one of the factors accounting for this phenomenon. High parity may be associated with breast cancer that is diagnosed in women younger than 45-50 years of age, but it appears to be protective against breast cancer diagnosed at older ages (Kelsey et al., 1993); thus the high parity of blacks than whites could be partly responsible for these risk differentials.

### **Incidence trends of breast cancer in the US**

Between 1973 and 1998, incidence rates of invasive breast cancer increased for women age 40 and over, although rates grew more than two-and-a-half times faster among women age

50 and older than for women in their 40s (Howe et al., 2001). Incidence rates for invasive breast cancer did not increase for women under age 40 during this time. Incidence rates of ductal carcinoma-in-situ (DCIS) increased for women of all ages during this same time period, although rates grew fastest in women over age 50 (Ernster et al., 1996). The perceptions of increasing numbers of breast cancer cases in young women in the late 1980s and early 1990s are largely due to the growth and aging of the US population, as many “baby boomer” women reached ages 25-40 at that time (Howe et al., 2001). Since 1985, breast cancer incidence rates among women under age 40 have actually declined significantly at an average 1.3% per year. It is important to note that in the past decade, incidence rates of breast cancer have remained relatively unchanged in women of all racial and ethnic groups.

There has been an important reduction in breast cancer death rates in the US in recent years beginning in the late 1990s (Howe et al., 2001). This decline in breast cancer mortality has been attributed to both improvements in breast cancer treatments and the benefits of mammography screening (Ries et al., 2001).

### **Incidence of Breast Cancer in African Populations**

Although there are no accurate data on the incidence rate of breast cancer in most of sub-Saharan Africa, data emerging from the few cancer registries within the region gives estimates of the incidence of the disease within various countries in the region. Reports from the Ibadan cancer registry in Nigeria estimated the incidence of breast cancer in Nigeria in 1976 to be 15.3 per 100,000 but this rose to 33.6 per 100,000 by 1993 (Ihekweba FN, 1992). The age standardized incidence rate (world standard population) from the Abijan cancer registry in Ivory Coast is 21.4 per 100,000 (Echimane et al, 2000). Other incidence figures from sub-Saharan

Africa are 20.4 per 100,000 for the Harare cancer registry in Zimbabwe (Chokunoga et al., 2000) and 16.4 per 100,000 from Kyadondo County in Uganda (Wabinga et al., 2000). Some other registries have reported lower incidence figures of 10.9 per 100,000 in Conarkry, Guinea (Koulibaly et al., 1997), 10.2 per 100,000 from Bamako Mali (Bayo et al., 1990) and 3.4 per 100,000 from Gambia cancer registry (Bah et al., 1990). Although the above incidence figures are much lower than the incidence of 79.3 per 100,000 in African American women in the U.S. (SEER, 1988-1992), there is general consensus that there is gross under-reporting due to low awareness, poverty, sociocultural factors and the absence of breast cancer-screening programs in countries within sub-Saharan Africa.

For the past two centuries, it has been suspected that sex hormones particularly estrogens may play some role in the etiology of breast cancer. It was, however, in the early 1970s that the role of these hormones in the causation of the disease was demonstrated by MacMahon et al. (1973). In 1983, Pike and colleagues (1983) observed that when the age-incidence curve was plotted on a log-log scale, the curve produced assumed a straight line until approximately age 50 years, when a decrease in the curve is noted, indicating that the premenopausal period probably creates a fertile period for the pathophysiological processes culminating in the manifestation of the disease. Since then, a lot of studies have been conducted in an attempt to explain the role of female hormones in the etiology and biological behavior of breast cancer. Several reproductive risk factors have been implicated in the etiology of the disease, including age at menarche and menopause, menstrual irregularity, age at first full-term pregnancy, parity, breastfeeding, and age at last childbirth. Other related hormonal factors include use of hormonal contraceptives, hormone replacement therapy and environmental exposure to hormone-related substances (xenoestrogens).

Although recent studies have provided evidence for familial clustering of breast cancer, high penetrance genes are thought to account for only 5-10% of all cases of the disease (Johnson et al., 1995), indicating that over 90% of cases of breast cancer may be accounted for by low-penetrance genes acting in concert with various environmental factors.

Cytochrome P4501A1 (CYP1A1) and cytochrome P4501B1 (CYP1B1) genes are involved in the hydroxylation of estradiol and estrone to catechol estrogen intermediates. Catechol estrogens particularly 4-hydroxyestradiol and 16-hydroxyestradiol have been shown to induce DNA damage via formation of estrogen catechol-DNA adducts as well as the generation of superoxide radicals that have been associated with single strand DNA breaks and other toxic effects on proteins and other cellular macromolecules (Han et al. 1994). On the other hand, the 2-hydroxy catechol estrogens including 2-OH estradiol and 2-OH estrone are devoid of estrogenic activity. COMT is one of the several phase II enzymes involved in the conjugation and inactivation of catechol estrogens. These enzymes are widely distributed in the body particularly in target organs prone to estrogen-induced carcinogenesis including the breast. In addition, different functional polymorphisms influencing the activity of these enzymes have been described and evidence is accumulating that these polymorphisms may determine inter-individual differences in exposure to estrogen-related carcinogenic metabolites.

In the past few decade efforts have been made to relate the above actions of estrogens and its metabolites in animal models to human breast carcinogenesis. Although studies in African American women have highlighted most of the reproductive risk factors for breast cancer in blacks, there is little data in the literature on the role of these variables in breast cancer susceptibility in African populations south of the Sahara. In the past decade, studies in molecular epidemiology have been devoted to quantifying the contribution of low-penetrance genes to



breast cancer risk in various populations. Most of these studies have been conducted in Caucasian populations, with few emerging reports in Asian populations. Very little literature exist on the role of these genetic polymorphisms on breast cancer risk in populations of African descent in the US, partly because of the low participation rate of African-American women in such studies. The few studies that have recruited African-American women often are of very small sample sizes. Overall, there has been a lot of inconsistency in the reported effects of these polymorphic variants on breast cancer risk in various populations partly because these polymorphisms have different allele frequencies in various populations. For example studies have shown that the G to A transition mutation in the COMT gene confer increased breast cancer risk in Asian populations (Yim et al., 2001, Huang et al., 1999) but not in most Caucasian populations (Millikan et al., 1998; Lavigne et al., 1997) studied to date despite the fact that the low-activity allele of the COMT gene has a much higher prevalence in Caucasian populations compared with Asian populations. In addition, some CYP1A1 polymorphic variants such as the CYP1A1 M1 is associated with increased risk of breast cancer in African-American women but not in Caucasians.

Overall, the existing literature on risk factors for breast cancer is inadequate particularly in populations of African descent. The present study, recruiting 250 Nigerian women with histologically confirmed breast cancer and 250 aged-matched control subjects, and aimed at evaluating the epidemiological and genetic risk factors for breast cancer in Nigeria women was designed to test the following hypothesis:

### **Hypothesis 1**

Women with breast cancer will have lifetime/reproductive experiences associated with higher estrogen exposure compared to those without the disease. We speculate that women with breast cancer will have earlier age at menarche, later age at menopause, later age at first full-term pregnancy, lower parity, and shorter overall duration of breastfeeding compared to women without the disease. In addition, we hypothesize that women with breast cancer will be more likely to have positive history of breast cancer in first- and second-degree relatives compared to the control subjects.

### **Hypothesis 2**

Women with breast cancer are more likely to harbor the low-activity COMT (Met) allele of catechol-O-methyltransferase (COMT), the gene encoding the phase II enzyme responsible for the detoxification of the carcinogenic catechol estrogens particularly 4-hydroxyestradiol to its biologically inactive intermediate, 4-methoxyestradiol for subsequent excretion. We speculate that this slower rate of detoxification might lead to the accumulation of genotoxic metabolites such as 4-hydroxyestradiol thereby exposing women with this variant allele to increased risk of breast cancer.

### **Hypothesis 3**

We also hypothesized that women with breast cancer will experience lower rate of 2-hydroxylation of estradiol compared to control women. This lower 2-hydroxylation rate is based on the speculation that these cancer-bearing women will harbor cytochrome P450 1A1 (CYP1A1) polymorphisms that encode enzymes with decreased catalytic activity. This will result in lower lifetime exposure to 2-hydroxyestradiol (a non-carcinogenic metabolite), which is

converted to 2-methoxyestradiol. 2-Methoxyestradiol has been shown to exert anti-angiogenic, anti-tubulin, and antiproliferative properties on tumor cells.

## **2. CASE-CONTROL STUDY OF RISK FACTORS FOR BREAST CANCER IN NIGERIAN WOMEN**

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## 2.1. ABSTRACT

This study was aimed at evaluating the risk factors for breast cancer in Nigerian women. A case-control design recruiting 250 women with breast cancer and 250 age-matched female controls was adopted for the study. Both cases and controls were drawn from four University Teaching Hospitals located in Midwestern and Southeastern Nigeria. Data on the clinical and epidemiological characteristics of the respondents were collected using interviewer-administered structured questionnaires followed by the anthropometric measurements. The mean ages of the cases and controls were 46.1 and 47.1 years, respectively. Fifty-seven percent of the cases were premenopausal while 43% were post- menopausal. Using conditional logistic regression, the effects of the various risk factors for breast cancer in the study population were assessed. Positive family history of breast cancer in first- and second-degree relatives was associated with a 15-fold increased risk of breast cancer [Odd ratio (OR) = 14.99, 95% Confidence interval (CI), 1.98, 113.47]. Also, waist to hip ratio (OR = 2.10, 95% CI 1.44, 3.06), history of abortion (OR = 2.83, 95% CI 1.12, 7.19), increasing age at first childbirth (OR = 1.39 95% CI 1.11, 1.73) and higher level of education (OR = 1.31, 95% CI 1.07, 1.61) conferred increased risk of breast cancer. Increasing parity (OR = 0.87, 95% CI 0.77, 0.99) and increasing duration of breastfeeding (OR = 0.75, 95% CI 0.62, 0.91) conferred protection against breast cancer. The findings from this study have shown that family history and reproductive characteristics are significant predictors of breast cancer risk in Nigerian women.

**Key words:** breast cancer, risk factors, Nigeria women.

## **2.2. INTRODUCTION**

Breast cancer is currently the most common malignancy in Nigerian women and the incidence seems to be on the increase<sup>1</sup>. It has overtaken carcinoma of the cervix and if the present trend is maintained, it will become, for Nigeria, and most other developing countries, the most important non-communicable disease of the new millennium.

The actual cause of breast cancer is unknown but studies have implicated age, gender, heredity, reproductive factors, diet and anthropometric characteristics as possible etiological factors. Most of the available literature on the role of these risk factors in breast susceptibility is drawn from Caucasian populations. While these factors may be at play in Nigerian women, it is important to note that there is considerable variation in the geographical, racial and ethnic distribution of the disease attributed to environmental and genetic factors<sup>2</sup>. Anecdotal observations by clinicians in Nigeria suggest that the epidemiological characteristics of the disease in Nigerian women differ from that in Caucasian populations. For example, most women with breast cancer in Nigeria are multiparous and they practice prolonged lactation, factors, which are thought to be protective against the disease.

While several investigators have reported the clinical and pathological characteristics of breast cancer in Nigerian women,<sup>3-6</sup> little is known about risk factors of the disease in this population<sup>3,7,8</sup>. Yet it is the nature of the disease that each society, race and population must seek to define the characteristics of the disease among its people and evolve appropriate management strategy. This study is aimed at identifying risk factors for breast cancer in Nigerian women. Identification of these factors may enhance the ability to prevent the disease by permitting better-focused health education and other preventive strategies.

## **2.3. METHODS**

### **2.3.1. Study population**

The study participants consisted of 250 breast cancer cases and 250 age- and sex-matched (within 5 years) controls recruited from four University Teaching Hospitals in Midwestern and Southeastern Nigeria including the University of Benin Teaching Hospital, Benin-City; University of Nigeria Teaching Hospital, Enugu; Nnamdi Azikiwe University Teaching Hospital, Nnewi; and University of Port Harcourt Teaching Hospital, Port Harcourt. The study protocol and consent forms were approved by the Institutional Review Boards of these four hospitals and the University of Pittsburgh. The cases consisted of prevalent and incident cases of breast cancer that were seen within these hospitals during the period of study. Breast cancer was defined as histologically confirmed malignant breast disease. Hospital-based, age-and sex-matched controls were recruited at the time of their outpatient clinic visits or in-patient wards at each of the hospitals where cases were recruited. The age match was within 1 to 5 years. Eligible controls were women who were being treated for non-hormonal and non-cancerous lesions.

### **2.3.2. Recruitment of study participants**

Physicians at the various study sites reviewed in-patient and outpatient medical records for information regarding past medical history and current medical complaint to identify potential study participants. Brief information about the study was provided to these potential study participants after which those willing to participate in the study were asked to contact the investigators for further information about the study.

### **2.3.3. Data collection**

Data was collected during one 30-minute visit. First, key details of the study in respect of objectives of the study, study protocol, risks and benefits, confidentiality and rights of participants were explained to all potential participants. Those willing to participate signed informed consent after which questionnaires were administered by the investigators. The interview was conducted in English; however when participants did not understand English, literate adult relatives, who as a rule accompany patients to the hospital, explained contents of the questionnaire and consent forms to the study participants. Each potential participant had an option to refuse participation in the study. An opportunity was also granted for participants to ask any questions.

The questionnaire was designed to gather demographic data including age, religion, educational and marital status and occupational history. Information in respect of use of alcohol, cigarette smoking, history of breast and other cancers in first- and second-degree relatives and position among siblings were also obtained. Reproductive characteristics related to age at menarche, age at first childbirth, duration of breastfeeding of each child, use of hormonal and surgical contraceptives, history of abortion, age at menopause and use of hormone replacement therapy were also noted. For case participants, age at diagnosis of breast cancer and treatment received were also noted. In addition knowledge of study participants about breast cancer was also obtained.



#### **2.3.4. Anthropometric measurements**

Height, weight, waist and hip measurements were taken while the subject was standing. The height was measured with a vertical tape attached to the wall; the weight was taken using a calibrated scale; the waist measurement was made at the umbilicus; and, the hips were measured at the widest part of the buttocks.

#### **2.3.5. Data analysis**

All questionnaires were reviewed for missing or incorrect data before the end of the interview section with each participant. All forms were reviewed for suspicious data, and returned to the participant's file to be confirmed or corrected the following day, if necessary. The data analysis was done using the Statistical Analysis System (SAS) software (Version 8.0). Descriptive analysis was carried out to characterize the demographic variables of the study participants. For logistic regression analysis, the variables were classified as follows: family history of breast cancer in first- and second-degree relatives (yes/no), waist/hip ratio dichotomized based on the median value in controls, abortion (yes/no), age at first childbirth (< 20 years, 20-24 years, 25-29 years, and  $\geq 30$  years), education (< 8 years, 8 through 11 years, 12 years or completed High School, Vocational/Technical Training, Some College, Completed College, and Postgraduate), parity (none, 1, 2, 3, 4, and  $\geq 5$ ), and duration of breastfeeding (1-12 months, 13-24 months, 25-36 months, 37-48 months, and  $\geq 49$  months). Other categorical variables that were entered into the logistic regression include cigarette smoking (yes/no), alcohol consumption (yes/no), regularity of menses (yes/no), use of hormone contraceptive pills (yes/no), age at menarche ( $\leq 12$  years, 13, 14, 15, 16, 17, and  $\geq 18$  years), and age at menopause ( $\leq 45$  years, 45-50 years, 51-55 years, and  $\geq 55$  years). Body mass

index (BMI), calculated as weight divided by the square of height ( $\text{kg/m}^2$ ) was dichotomized based upon the median value of BMI among the controls ( $24.65 \text{ kg/m}^2$ ).

Conditional logistic regression was used to assess the strength of the association between each of the hypothesized risk factors and breast cancer risk. Each matched case was paired with the corresponding control to enable differences between the cases and controls to be calculated. First each variable was assessed alone. The strength of significant variables were further assessed by building multivariate models.

## **2.4. RESULTS**

### **2.4.1. Socio-demographic characteristics of the study population**

#### **Age distribution**

There were 250 cases (all females) of histologically confirmed breast cancer and 250 aged- and sex-matched controls that were recruited for this study. The participation rate in this study was very high as less than 1% of the patients were excluded from the study. Reasons for exclusion included refusal to allow a blood draw and patient's unwillingness to participate in the study. The age at diagnosis for the breast cancer cases ranged from 17 years to 95 years with a mean of  $46.1 \pm 12.6$  years. The peak age of the cases were in the 45-49 year age range [43 patients (17.2%)], closely followed by the 40-44 year age range [41 patients (16.4%)]. Eighty patients (32.0%) were below the age of 40 years while 22 patients (8.8%) were aged 65 years and older as shown in Figure 2-1. The mean age of the controls was 47.1 years.

#### **Educational status**

Fewer cases [57 (22.8%)] than controls [91 (36.4%)] had less than eight years of education while more cases [136 (54.4%)] than controls [113 (45.2%)] completed High School

or post High School education. In a conditional logistic regression model controlling for age, higher level of education (completed High School or post High School education) conferred a significant 31% increased risk of breast cancer (OR = 1.31, 95% CI 1.07, 1.61) as shown in Table 2-1.

### **Marital status**

Majority of the cases [195 (78.0%)] and controls [190 (76.0%)] were married or living as married. Fewer cases [24 (9.6%)] than controls [36 (14.4%)] were never married. There were more women who had divorced among the cases [10 (4.0%)] compared with the controls [2 (0.8%)]. Two-third of the patients (67%) presented in hospital with advanced stages of breast cancer (Manchester Stages III and IV).

### **Usual adult occupation**

The majority of the study participants were engaged in trading; 82 cases (32.8%) versus 100 controls (40.0%). Most of the traders were engaged in petty trading involving sale of food items and domestic wares. Slightly more cases [81 (32.4%)] than controls [74 (29.6%)] were employed in the public service. The most common jobs in the public service included teaching, secretarial duties and nursing. Similar proportions of cases and controls were engaged in farming at a subsistence level usually involving cultivation of food crops. The other less common occupations included catering, fashion designing and hairdressing.

### **Family history of cancer**

A much higher number of cases (19) than controls (6) reported positive family history of various types of cancer. More cases (15) than controls (1) reported family history of breast cancer. Six of the family breast cancers in the cases were in first-degree relatives, comprising four in sisters and two in mothers while seven were in second degree relatives consisting of four in aunts, one in a grandmother, one in a cousin and one in a niece. Two of the cases reported history of breast cancer in two relatives; one was that of breast cancer in her sister and grandmother while the second reported history of breast cancer in her sister and cousin. The only control with positive family history of breast cancer was reported in a sister. Other cancers reported in family members in the cases include one case each of carcinoma of the cervix, carcinoma of the prostate, and one patient that reported history of liver cancer in her father and brother. The other cancers reported by control participants include two cases of squamous cell carcinoma of the skin, one case of oral cancer and one case of carcinoma of the larynx. There was one case of an unspecified abdominal cancer in a relative of one of the controls. As shown in Table 2-1, family history of breast cancer was strongly associated with a 15-fold increased risk of breast cancer (OR = 14.99, 95% CI 1.98, 113.47) in a conditional logistic regression age.

### **Use of alcohol**

Alcohol consumption was slightly more common among the cases [83 (33.2%)] compared with the controls [70 (28.0%)]. Most of them were occasional drinkers and the types of alcohol consumed include beer, palm-wine, and locally brewed gin. Alcohol consumption was

associated with a non-significant 29% increased risk of breast cancer among the study population (OR = 1.29, 95% CI 0.87, 1.90) as shown in Table 2-2.

### **Cigarette smoking**

Cigarette smoking was not a common practice among study participants as only two of the cases and two of the controls reported having smoked cigarettes regularly for more than six months. Cigarette smoking was not associated with increased risk of breast cancer.

## **2.4.2. Reproductive characteristics**

### **Age at menarche**

The age at menarche in cases (range 10-20 years and mean 14.75 years) did not differ significantly from that of controls (range 10-20 years, mean 14.5 years). As shown in Table 2-2, later age at menarche (>14 years) was associated with a non-significant 11% increased risk of breast cancer (OR = 1.11, 95% CI 0.98, 1.26).

### **Age at first childbirth**

There were 210 parous women among the cases and 209 parous women among the controls. The age at first childbirth ranged from 14-44 years (mean 23.18 years) for the cases and 14-42 years (mean 21.87 years) for the control participants. Fewer cases [48 (22.8%)] had their first childbirth before the age of 20 years compared with controls [73 (34.93%)]. More cases [29 (13.81%)] than control participants [13 (6.22%)] had their first childbirth after the age of 30 years. Older age at first childbirth was associated with a significant 39% increased risk of breast cancer in a model controlling for age (OR = 1.39, 95% CI 1.11, 1.73).

### **Duration of breastfeeding**

All parous women among the study participants breastfed their babies ranging from 2-216 months (mean 65.49 months) among the cases and 2-312 months for the controls (mean 80.96 months). More cases [93 (44.50%)] than controls [68 (32.69%)] breastfed for 48 months and below while fewer cases [116 (55.50%)] than control participants [140 (67.31%)] breastfed for over 48 months. Breastfeeding was associated with a significant 25% reduction in breast cancer risk (OR = 0.75, 95% CI 0.62, 0.91).

### **Parity**

The parity of the cases ranged from 0-11 with a mean parity of 4 while parity among the controls ranged from 0-13 with a mean of 5. More cases (97) than controls (69) had five children and below while fewer cases (113) than controls (140) had more than five children. Higher parity was associated with a significant 12% reduced risk of breast cancer (OR = 0.88, 95% CI 0.81, 0.96).

### **Age at menopause**

About equal number of cases [107 (42.8%)] and controls [108 (43.2%)] were postmenopausal. Fewer cases (11) than controls (17) attained menopause before the age of 45 years while more cases (48) than controls (38) became menopausal between the ages of 45 and 49 years. Slightly fewer cases (41) than controls (47) attained menopause between the ages of 50 and 54 years. About equal number of cases (7) and controls (6) attained menopause after the age

of 55 years. Older age at menopause was associated with a 7% non-significant increased risk of breast cancer (OR = 1.07, 95% CI 0.71, 1.60).

### **Regularity of menses**

Information about menstrual regularity was available in 227 cases, of which 27 (11.89%) reported irregular menses. Among the controls 231 provided information about menstrual regularity and 31 of them (13.42%) had irregular menses. Irregular menses was associated a 19% non-significant reduced risk of breast cancer (OR = 0.81, 95% CI 0.45, 1.44).

### **Use of hormonal contraceptives**

Use of hormonal contraceptives was reported among 43 (17.2%) of the cases and 32 (12.8%) control participants. Hormone contraceptive use conferred a 40% non-significant increased risk of breast cancer among study participants (OR = 1.40, 95% CI 0.84, 2.34).

### **Birth order**

Information about birth order was available in 222 cases and 233 controls. The birth order of the cases ranged from 1-9 while the range is from 1-11 for the controls. About equal number of cases (136) and controls (135) were in the 1-3 birth order category while slightly fewer cases (68) than controls (77) were in the 4-6 category. Birth order was not associated with increased of breast cancer in the study population.

### **2.4.3. Anthropometric measurements**

#### **Body mass index (BMI)**

One hundred and thirty-six cases (54.4%) and 125 controls (50.0%) had BMI below 24.65. Slightly fewer cases [114 (45.6%)] than controls [125 (50.0%)] had BMI above 24.65 (the median BMI for controls). Body mass index was not associated with increased risk of breast cancer (OR = 0.83, 95% CI 0.58, 1.19).

#### **Waist/Hip ratio**

Median waist to hip ratios for the cases and controls were 0.92 and 0.90 respectively. Fewer cases [89 (35.6%)] than controls [133 (53.2%)] had waist/hip ratio of 0.90 and below while more cases [161 (64.4%)] than controls [117 (46.8%)] had waist/hip ratio above 0.90. Higher waist/hip ratio was associated with a 2.0 fold increased risk of breast cancer in a logistic regression model (OR = 2.0, 95% CI 1.39, 2.87) as shown in Table 2-1.

### **2.4.4. Controlling for additional risk factors**

Significant predictors of breast cancer identified in the univariate conditional logistic regression model including family history of breast cancer, waist/hip ratio, abortion, education, marital status, age at first childbirth, parity and duration of breastfeeding were entered into the model and various combinations of risk factors were found to be significant as shown in Table 2-3. Overall, family history of breast cancer, waist/hip ratio, age at first childbirth, duration of breastfeeding, and education remain significant with three variables in the model while family



history of breast cancer, waist/hip ratio, age at first childbirth and education were retained in the model controlling for four additional factors.

#### **2.4.5. Analysis of menopausal status**

Further stratified analysis on the basis of menopausal status revealed that educational status (OR 1.45; 95% CI 1.09, 1.95), use of hormone contraceptives (OR 2.67; 95% CI 1.04, 6.82), and late age at first childbirth (OR 1.72; 95% CI 1.16, 2.53), were associated with significantly increased risk of breast cancer risk in postmenopausal women but not in premenopausal women. Longer duration of breastfeeding conferred a 33% reduced risk of breast cancer also in postmenopausal women (OR 0.67; 95% CI 0.48, 0.92). In premenopausal women, higher waist to hip ratio was associated with a significant 4-fold increased risk of breast cancer.

## **2.5. DISCUSSION**

This study was aimed at examining the role of reproductive and other epidemiological risk factors in susceptibility to breast cancer among women in Midwestern and Southeastern Nigeria. We were interested in this subject because of the paucity of data on risk factors for breast cancer in sub-Saharan Africa. Establishing the risk factors of the disease is the first major step in understanding the etiology of the disease and designing appropriate control and preventive measures. Much of what has been reported about risk factors for breast cancer has come from studies conducted in populations in the other parts of the world. It is known that environmental factors may play considerable role in breast cancer susceptibility and most of the populations studied live in different geographical areas. We were therefore interested in

assessing the risk factors that may be at play in a black population in Midwestern and Southeastern Nigeria. This population provides a particularly interesting environment for this study because of the very high population density in Midwestern and Southeastern Nigeria<sup>9</sup>, with a very high participation rate of over 99%. This is in contrast to the findings of other investigators who have reported low recruitment rates particularly for blacks in the United States<sup>10</sup>.

The age of the breast cancer patients in this study ranged from 17 to 90 years with a mean of  $46.1 \pm 12.6$  years. Fifty-seven percent of the cases were premenopausal while 43% were postmenopausal and majority of the patients presented in hospital with advanced stages of the disease. The disease pattern in this study reflects the general picture in most sub-Saharan African populations. The mean age of in the cases in this study is slightly higher than that reported by Anyanwu<sup>4</sup> in Eastern Nigeria (44 years), slightly lower than reports from Ibadan in Western Nigeria (48 years)<sup>3</sup> but much lower than figures among blacks patients in Durban, South Africa ( $54 \pm 10.9$  years)<sup>11</sup> and Chris Hani Baragwanath Hospital in Soweto (50 years)<sup>12</sup>. Thus, in sub-Saharan African countries breast cancer occurs at a much earlier age than in the case of patients in most developed populations. Interestingly, African-American patients also tend to present at a younger age. In one study, 33% were under 50 years of age, compared with 25% in the case of white patients studied<sup>13</sup>. The proportion of postmenopausal women among the cases in this study is higher than the 20% previously reported from Ibadan in Nigeria<sup>3</sup> but considerably less than two-thirds reported in Caucasians<sup>14,15</sup>.

Among the relevant etiological factors identified in this study, family history of breast cancer in first- and second-degree relatives conferred a 15-fold increased risk of breast cancer, controlling for age but this risk was attenuated to eight-fold when additional factors including

waist/hip ratio, age at first childbirth and education were added to the model. About 30.4% of breast cancer cases in this study fell within the category of familial and hereditary breast cancer using the criteria of Lynch and associates.<sup>15</sup> This is in keeping with reports in other populations with detailed characterization of pedigree suggesting that familial and hereditary breast cancer constitute about 32% of the total incidence of breast cancer.<sup>16</sup>

Waist/hip ratio, a surrogate marker for central adiposity was associated with a 2-fold increased risk of breast cancer in this study and it remained significant in premenopausal women when participants were further stratified by menopausal status. Epidemiological studies of body fat distribution using waist/hip ratio have produced contradictory results; some being positively related breast cancer,<sup>6,17-21</sup> others showing no association;<sup>22,23</sup> inconsistencies in these findings being related to differences in study design and sample size. Abdominal obesity is linked to hyperinsulinemia in both pre- and post-menopausal women and estrogen production is increased in the presence of abdominal obesity<sup>24,25</sup>. Increased estrogen bioactivity is thought to be related to increased estrogen production in fat deposits and also to decreased estrogen binding because of decreased levels of sex hormone binding globulin and increased triglyceride levels. Women with abdominal obesity also show an increase in unbound testosterone levels and may in addition show an increased production of testosterone and dihydrotestosterone.<sup>26</sup> Insulin levels affect plasma lipid levels, and dyslipidemia is increased in the presence of abdominal obesity. The relative importance of abdominal obesity and hyperinsulinemia is uncertain in relation to a role in mammary carcinogenesis, but in a subset of women, the metabolic/endocrine concomitants of hyperinsulinemia associated with changes in the bioactivity of insulin-like growth factors (IGFs) in breast tissue, might act synergistically with increased estrogen bioactivity<sup>27</sup>.

A history of abortion was associated with increased risk of breast cancer among women in this study and this is consistent with emerging consensus on the increased risk of breast cancer particularly in women who had induced abortion after the first trimester of pregnancy. Since Segi et al<sup>28</sup> provided epidemiological evidence for an association between induced abortion and breast cancer risk in 1957, several reports have appeared in the literature that showed either no risk or an elevated risk for breast cancer following induced abortion. In 1980, Russo and Russo<sup>29</sup> provided experimental evidence of the mechanism responsible for the protective effect of early full term pregnancy, the abrogation of which is one way induced abortion increases breast cancer risk. After an extensive and detailed meta-analysis of the existing literature on the subject, Brind et al<sup>30</sup> noted that a significant positive association exists between induced abortion and breast cancer risk, independent of the effect an induced abortion has in delaying first full term pregnancy. Moreover, the increased risk is seen in both prospective and retrospective studies from around the world, in populations with the widest imaginable differences in ethnicity, diet, socioeconomic and lifestyle factors and which differ in many aspects of design, and whose data extend over more than half a century in time<sup>30</sup>. This finding is consistent with the existing knowledge on human biology, oncology and reproductive endocrinology, and supported by a coherent body of laboratory data as well as epidemiological data on other risk factors involving estrogen excess, all of which together point to a plausible and likely mechanism by which the surging estradiol of the first trimester of pregnancy, if it is aborted, may significantly add to a women's breast cancer risk.

Our study has demonstrated the protective effect of early age at first childbirth, increasing duration of breastfeeding and parity on breast cancer risk. First full-term pregnancy before age of 18 years reduces the risk of breast cancer and the risk is significantly higher in women with first

full term pregnancy after the age of 35 years<sup>31</sup>. Most studies have also found that for first births over the entire childbearing period, the higher a woman's age at first birth, the higher the risk<sup>32</sup>. While some studies have reported no protective effect for early age at first full term pregnancy others have found that age over 30 years at first child birth was associated with an increased risk of breast cancer relative to nulliparous women<sup>33</sup>.

The effect of parity on breast cancer risk is not clearly understood. In most studies, high parity is found to be associated with low rates of breast cancer, but the extent to which this relationship can be explained by an inverse association between parity and age at first birth varies between studies<sup>34</sup>. Several studies have reported a protective effect of parity independent of the effect of age at first full term pregnancy. Kvale et al<sup>35</sup> found a consistent and highly significant inverse association between high parity and breast cancer. The apparent protective effect of high parity was found in all subgroups of the patients according to demographic variables and could not be explained by other reproductive factors. There appears to be consistency in this finding across studies conducted in both high-risk, intermediate-risk and low-risk areas. The protective effect of parity seems stronger in postmenopausal than in premenopausal women, possibly on account of the confounding effect of time since last birth in younger women.

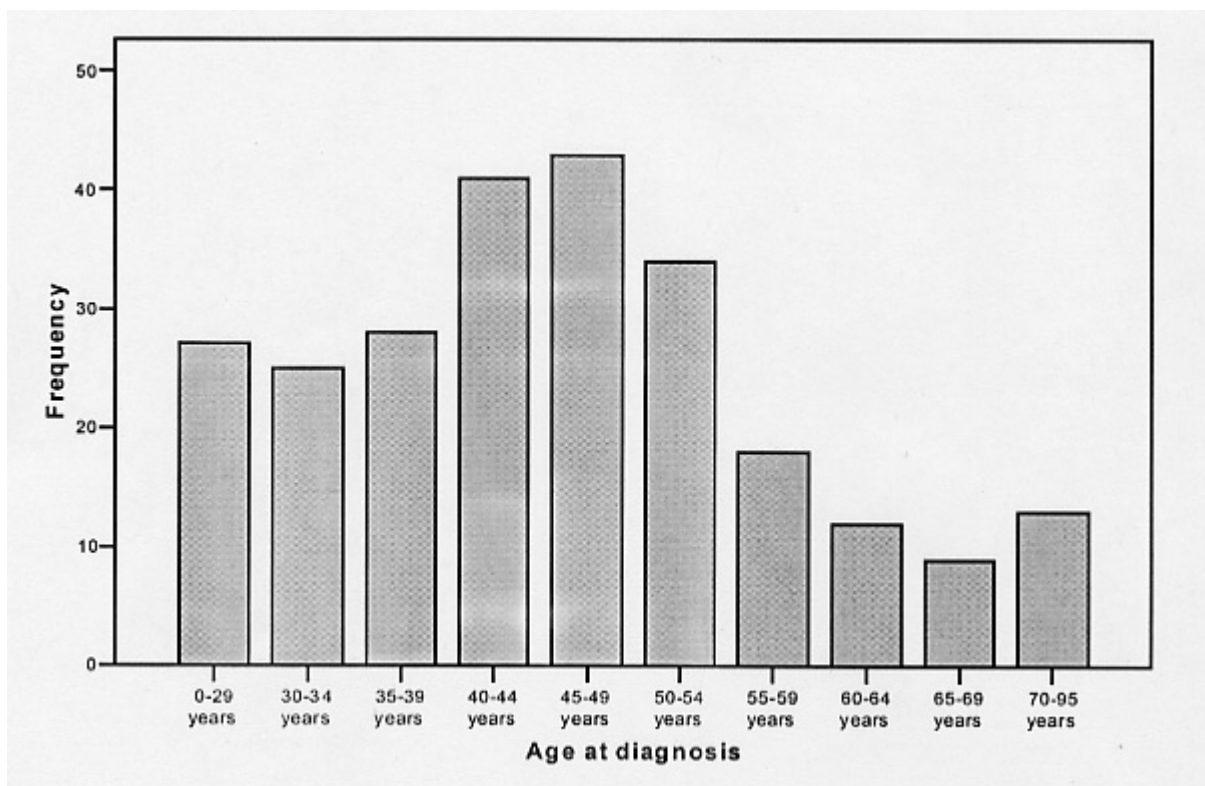
The long-term protective effects of pregnancy are contrasted with the observation that the risk of carcinogenesis is actually increased in the short term after a pregnancy<sup>36</sup>. It is known that hormones induce carcinogenesis by inducing cell proliferation, which is an essential component of carcinogenesis. This hypothesis is consistent with the observation that increased cell proliferation results in a larger pool of cells that are susceptible to defective DNA repair. This in turn leads to mutations, which are subsequently propagated through increased mitotic activity

present in proliferating cells, and can result in cancer formation. However, it has been shown that full term pregnancy induces differentiation of cells in the terminal duct lobular unit (TDLU) in the breast and this effect produces the long term effect of slowing the cell cycle in the epithelial cells of this location, which allows more time for DNA repair, which in turn will lead to decreased carcinogenesis<sup>37</sup>.

A number of epidemiological studies have investigated the relationship between breastfeeding and breast cancer risk. Our finding of a 25% reduction in risk conferred by breastfeeding is consistent with reports in the literature. Overall, studies suggest a 20-30 percent reduction in risk among women who have ever breastfed<sup>38</sup>. More consistently, a longer duration of breastfeeding has been associated with breast cancer risk reductions as great as 40-60 percent<sup>39</sup>. Recently, age at first lactation has been identified as the arbiter of risk, with an earlier age at initiation of lactation being associated with a stronger reduction in risk for premenopausal women and possibly for postmenopausal women<sup>40</sup>. However, because of the very strong correlation between age at first birth and age at first lactation, the independent effect of age at first lactation is difficult to isolate. It is notable that in countries with low risk of breast cancer, the protection conferred by lactation appears to be stronger and to be sustained throughout the postmenopausal period as well.

Higher education conferred an increased risk of breast cancer in this study, in keeping with reports in the literature<sup>41</sup>. Although, we did not measure socioeconomic status, an established risk factor for breast cancer in most studies<sup>42,43</sup>, education is a strong surrogate for socioeconomic status. The quest for higher education delays age at marriage and age at first childbirth and it is associated with reduced parity and reduced duration of breastfeeding; these factors have been shown to reduce breast cancer risk.

In conclusion, this study has examined the relationship between various factors and breast cancer risk in Nigerian women. It has demonstrated increased breast cancer risk associated with family history of breast cancer, abdominal adiposity, abortion and higher education and the reduced risk conferred by various reproductive variables; these findings are consistent with reports in the literature. However, studies with larger sample sizes are recommended for better characterization of the role of these risk factors in breast cancer risk in Nigerian women. This will provide an enabling framework for developing breast cancer risk assessment tools for the population with the aim of identifying high-risk individuals for primary and secondary prevention.



**Figure 2-1: Age distribution of breast cancer patients**



**Table 2-1: Conditional logistic regression comparing cases and controls. Significant predictors of breast cancer risk [Numbers (N), Percentages (%)], [Means (S.D.)], age-adjusted odds ratio, 95% confidence interval for cases and controls**

Variable		Cases	Control	Odds ratio	95% CI
Family history of breast cancer	Yes	15 (6.00) <sup>a</sup>	1 (4.00) <sup>a</sup>	14.99	1.98,113.47
	No	235 (94.00) <sup>a</sup>	249 (96.00) <sup>a</sup>	1.00	
Waist/hip ratio (>0.90)	Yes	161 (64.40) <sup>a</sup>	117 (46.80) <sup>a</sup>	2.00	1.39,2.87
	No	89 (35.60) <sup>a</sup>	133 (53.20) <sup>a</sup>	1.00	
Abortion	Yes	18 (7.20) <sup>a</sup>	7 (2.80) <sup>a</sup>	2.43	1.01,5.85
	No	232 (92.80) <sup>a</sup>	243 (97.20) <sup>a</sup>	1.00	
Age at first full-term pregnancy		23.18 (5.36) <sup>b</sup>	21.87 (4.74) <sup>b</sup>	1.39	1.11,1.73
Education >= High School	Yes	136 (54.40) <sup>a</sup>	113 (45.20) <sup>a</sup>	1.31	1.07,1.61
	No	114 (54.60) <sup>a</sup>	137 (54.80) <sup>a</sup>	1.00	
Parity		4.1 (2.9) <sup>b</sup>	4.7 (3.1) <sup>b</sup>	0.88	0.81,0.96
Duration of breastfeeding		65.48 (47.32) <sup>b</sup>	80.96 (53.67) <sup>b</sup>	0.75	0.62,0.91

**Table 2-2: Conditional logistic regression comparing cases and controls. Non-significant predictors of breast cancer risk [Numbers (N), Percentages (%)], [Means (S.D.)], age-adjusted odds ratio, 95% confidence intervals**

Variable		Cases	Control	Odds ratio	95% CI
Cigarette smoking	Yes	2 (0.80) <sup>a</sup>	2 (0.80) <sup>a</sup>	1.00	0.14,7.01
	No	248 (99.20) <sup>a</sup>	248 (99.20) <sup>a</sup>	1.00	
Alcohol consumption	Yes	83 (33.20) <sup>a</sup>	70 (28.00) <sup>a</sup>	1.29	0.87,1.90
	No	167 (66.80) <sup>a</sup>	180 (72.00) <sup>a</sup>	1.00	
Irregular menses	Yes	27 (11.89) <sup>a</sup>	31 (13.42) <sup>a</sup>	0.81	0.45,1.44
	No	200 (66.11) <sup>a</sup>	200 (86.58) <sup>a</sup>	1.00	
Use of hormonal contraceptives	Yes	43 (17.27) <sup>a</sup>	32 (12.85) <sup>a</sup>	1.40	0.84,2.34
	No	206 (82.73) <sup>a</sup>	217 (87.15) <sup>a</sup>	1.00	
Age at menarche		14.75 (1.73) <sup>b</sup>	14.50 (1.60) <sup>b</sup>	1.11	0.98,1.26
Age at menopause		48.72 (3.27) <sup>b</sup>	48.48 (3.90) <sup>b</sup>	1.07	0.71,1.60
Height		163.49 (6.81) <sup>b</sup>	162.98 (6.54) <sup>b</sup>	1.01	0.98,1.04
Weight		65.78 (15.68) <sup>b</sup>	66.23 (13.72) <sup>b</sup>	0.99	0.98,1.01
BMI >24.65	Yes	114 (54.60) <sup>a</sup>	125 (50.00) <sup>a</sup>	0.87	0.67,1.12
	No	136 (54.40) <sup>a</sup>	125 (50.00) <sup>a</sup>	1.00	

**Table 2-3: Multivariate conditional logistic regression comparing cases and controls.****Significant predictors of risk**

Variable	Odd ratio (OR)	95% Confidence Interval (CI)
Family history of breast cancer	8.97	(1.12, 71.66)
Waist/Hip ratio	1.90	(1.24, 2.93)
Age at first childbirth	1.45	(1.13, 1.86)

Variable	Odd ratio (OR)	95% Confidence Interval (CI)
Family history of breast cancer	9.44	(1.17, 76.03)
Waist/Hip ratio	2.00	(1.30, 3.09)
Duration of breastfeeding	0.77	(0.63, 0.95)

Variable	Odd ratio (OR)	95% Confidence Interval (CI)
Family history of breast cancer	11.66	(1.50, 90.48)
Waist/Hip ratio	2.01	(1.38, 2.93)
Education	1.31	(1.62, 1.64)

Variable	Odd ratio (OR)	95% Confidence Interval (CI)
Family history of breast cancer	8.07	(1.003, 64.95)
Waist/Hip ratio	1.98	(1.27, 3.10)
Age at first childbirth	1.31	(1.01, 1.71)
Education	1.33	(1.05, 1.74)

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### **3. ASSOCIATION OF CATECHOL-O-METHYLTRANSFERASE (COMT) GENE AND BREAST CANCER RISK IN NIGERIAN WOMEN**

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### 3.1. ABSTRACT

Life-long estrogen exposure has been recognized as a predictor of breast cancer risk in women. Since polymorphisms in candidate genes involved in estrogen metabolism may contribute to determining life-time exposure to estrogen and its biologically diverse metabolites, we utilized a polymerase chain reaction (PCR)-based restriction fragment length polymorphism (RFLP) assay to assess the relationship between a G to A transition polymorphism in the catechol-O-methyltransferase (COMT) gene and breast cancer risk in a case-control study recruiting 250 Nigerian women with breast cancer and their age-matched controls. The frequencies of the COMT (Val/Val), COMT (Val/Met) and COMT (Met/Met) genotypes in the control subjects were 0.53, 0.40 and 0.07 respectively. In the final multivariate logistic regression model in all women, carrying at least one of low-activity allele of the COMT gene (COMT [Val/Met] + COMT [Met/Met]) was associated with a significant 33% reduced risk of breast cancer (OR = 0.57, 95% CI = 0.36-0.91). In premenopausal women, harboring at least one low- activity COMT (Met) allele conferred a non-significant 31% reduced risk of breast cancer (OR = 0.69, 95% CI 0.40-1.18) while there was a 14% reduced risk of postmenopausal breast cancer (OR = 0.86, 95% CI 0.46-1.61) for carrying at least one low-activity COMT (Met) allele. Our results suggest that harboring the COMT polymorphism appears to confer some protection against breast cancer risk in this population. To the best of our knowledge, this is the first study evaluating the association between this genotype and breast cancer risk in indigenous African populations. We therefore suggest more studies to further assess the contribution of this polymorphism to breast cancer susceptibility in sub-Saharan African populations.

### 3.2. INTRODUCTION

The standard mechanistic paradigm of estrogen-mediated carcinogenesis via estrogen receptor  $\alpha$ -induced cell proliferation<sup>1,2</sup> has been expanded to encompass emerging research data supporting a complementary genotoxic pathway mediated by the generation and redox cycling of reactive oxygen species through the metabolic effects of estrogen metabolites such 4-, and 16 $\alpha$ -hydroxy catechols and the estrogen quinones that result from the oxidation of catechols<sup>3,4</sup>. This paradigm shift is necessitated by evidence of estrogen-induced carcinogenesis in several animal models including the Syrian hamster kidney cells<sup>5,6</sup>, the uterus of CD-1 mice<sup>7</sup>, mouse mammary gland<sup>8</sup>, and pituitary of rats<sup>9</sup> following exposure to these estrogen metabolites.

Although several enzymes have been implicated in the hepatic and extrahepatic hydroxylation of endogenous estrogens in animals and humans, attention has focused on the 2-, 4-, and 16-hydroxylation pathways because of the known actions of metabolites in these pathways in both humans and animals. 4-Hydroxyestradiol has been shown to exhibit strong carcinogenic activity in several animal models including the male Syrian hamster kidney<sup>5,6</sup> and rat pituitary<sup>9</sup>. 4-Hydroxyestradiol carcinogenicity has been attributed to its ability to bind the classical estrogen receptor<sup>10</sup>, undergo metabolic redox cycling<sup>11</sup> to generate superoxide radicals and chemically-reactive quinone and semiquinone intermediates which damage DNA and other cellular macromolecules<sup>12</sup>, induce cell transformation and initiate tumorigenesis<sup>4</sup>. On the other hand, the 2-hydroxyl metabolites including 2-hydroxyestradiol and 2-hydroxyestrone lack carcinogenic activity and in fact, 2-methoxyestradiol (a product of subsequent O-methylation of 2-hydroxyestradiol) is a very potent inhibitor of tumor cell proliferation<sup>13</sup> and angiogenesis<sup>14</sup>.

The recent finding that chronic administration of quercetin (a substrate and inhibitor of catechol-O-methyltransferase) to male Syrian hamsters significantly increased the severity of estradiol-induced kidney tumors<sup>15</sup>, and the correlation of this effect with inhibition of enzymatic O-methylation of catechol estrogens during quercetin administration<sup>16</sup> suggest that factors that decreased the rate of O-methylation (mediated by catechol-O-methyltransferase [COMT]) of estrogen catechols might increase the rate of breast and other tumors in both animals and humans. In addition recent studies have shown that there are marked person-to-person variations in catechol-O-methyltransferase activity in red blood cells<sup>17</sup> and liver samples<sup>18</sup>, and the distribution of catechol-O-methyltransferase activity in the American population appears to follow a polymorphic bimodal pattern<sup>19</sup>. All these led to the hypothesis that women harboring the low-activity allele of the COMT gene might have a higher risk of estrogen-associated breast cancer due to decreased formation of antitumorigenic 2-methoxyestradiol and retarded inactivation of catechol estrogen intermediates (particularly 4-hydroxyestradiol which is hormonally active and potentially genotoxic).

Available data from association studies conducted in predominantly Caucasian<sup>20-23</sup> and Oriental populations<sup>24,25</sup> have shown inconsistencies with some demonstrating some increased risk with the low-activity allele while others show some protection against breast cancer risk in women carrying the low-activity alleles. There are few reports on the role of this genetic variant in populations of African descent mainly among African American women and most of these studies suffer from inadequate sample size with the risk of spurious associations. To the best of our knowledge, there are no reports on the role of this genetic variant on breast cancer risk in sub-Saharan African populations. This case-control study was designed to examine the

association between the G to A transition mutation in codon 158 of the COMT gene and breast cancer risk in Nigerian women, a population of indigenous African women.

### **3.3. MATERIALS AND METHODS**

#### **3.3.1. Subjects**

All the subjects for the study were recruited from four University Teaching Hospitals located in Midwestern and Southeastern Nigeria, including University of Benin Teaching Hospital, Benin City; Nnamdi Azikiwe University Teaching Hospital, Nnewi; University of Nigeria Teaching Hospital, Enugu; and University of Port Harcourt Teaching Hospital, Port Harcourt. Study protocols were approved by the Ethics and Research Committees of the Nigerian hospitals and the Institutional Review Board (IRB) of the University of Pittsburgh. Recruitment was conducted between September 2002 and April 2004. Two hundred and fifty women with breast cancer and 250 age- and institution-matched controls were recruited during the period of study. Cases consisted of women with histologically confirmed breast cancer and were recruited during surgical out-patient clinic visits or in-patient hospitalizations for treatment of breast cancer during the period. Control subjects were women attending surgical outpatient clinics or hospitalized for non-malignant surgical diseases such as road traffic accident and other injuries, abdominal conditions such as intestinal obstructions, inflammatory disorders such as cholecystitis, choledocholithiasis and appendicitis and urological diseases such as urolithiasis and urinary tract infections. Patients being managed for malignant diseases or hormonal disorders were excluded from the study. About 0.5% of eligible patients refused sample donation and were also excluded from the study.

Patient recruitment was carried out in the outpatient surgical clinics and surgical wards of the participating Nigerian hospitals. Written informed consent was obtained from study participants after detailed explanation of key points of the study including study objectives, risks and benefits, confidentiality and the rights of participants. Data was collected using interviewer-administered questionnaires targeted at demographic history including age, sex, religion, occupation, exposure to chemical fertilizers and pesticides, and rearing of domestic animals. Obstetric and gynecological history including age at menarche, age at first full-term pregnancy, parity, breastfeeding, age at menopause (for postmenopausal subjects), history of use of hormonal contraceptives and hormone replacement therapy and surgical oophorectomy. Information about social habits such as cigarette smoking and alcohol consumption was also obtained. Anthropometric measurements including height, weight, waist and hip circumference were taken at the end of the interview.

### **3.3.2. Sample donation and preparation**

Thirty milliliters of whole blood was collected in two 15 ml plain vacutainers and 10ml of whole blood was also collected in one 10 ml K<sub>3</sub> EDTA vacutainer tube from each of the study participants. The blood was stored in ice packs and centrifuged within 10 h of collection. Serum and clots from the plain vacutainer tubes were separated; the serum was aliquoted into ten 2 ml tubes while the clot was transferred into two plain 20 ml tubes. Samples in the K<sub>3</sub> EDTA vacutainer tubes were also centrifuged and buffy coat collected and stored in 3 ml tubes. All the samples were stored at -20 °C in the various study sites in Nigeria and later transferred to the Nigerian coordinating center in Polar Pack -20 °C ice packs for frozen shipments where the samples were stored at -20 °C and later shipped to University of Pittsburgh in dry ice using

FEDEX services. Samples were stored at  $-70^{\circ}\text{C}$  at the University of Pittsburgh until DNA extraction.

DNA extraction from buffy coats (and clots for subjects with no buffy coats) was carried out using QIAamp DNA Mini Kits (for buffy coats) and QIAamp DNA Midi Kits (for blood clots) protocols<sup>26,27</sup>. The extracted DNA was stored at  $4^{\circ}\text{C}$  until used for PCR and RFLP analysis.

### **3.3.3. PCR and RFLP analysis**

Genomic DNAs from the cases and control subjects were analyzed for the presence of the G to A transition polymorphism in the COMT gene by a PCR-based Restriction Fragment Length Polymorphism (RFLP) assay. PCR amplification of a 323 bp fragment of the COMT gene, including part the part of the exon 4 that contains the polymorphism was carried out using forward primer: CCT GCT CTT TGG GAG AGG T and reverse primer: GTC TGA CAA CGG GTC AGG TA. A 50  $\mu\text{l}$  PCR reaction mixture containing 2  $\mu\text{l}$  of genomic DNA, 8  $\mu\text{l}$  of deoxynucleotide triphosphates, 1  $\mu\text{l}$  each of forward and reverse primers, 5  $\mu\text{l}$  of 10X buffer, 1.5  $\mu\text{l}$  of MgCl and 0.2  $\mu\text{l}$  of Taq polymerase was placed in a MJ Research DYAD thermocycler. After denaturing for 5 min at  $95^{\circ}\text{C}$ , the DNA was amplified for 35 cycles at  $95^{\circ}\text{C}$  for 30 s,  $58^{\circ}\text{C}$  for 30 s, and  $72^{\circ}\text{C}$  for 30 s, followed by a 5 min extension at  $72^{\circ}\text{C}$ . A positive control containing genomic DNA (University of Pittsburgh Molecular Epidemiology Laboratory control) and a negative control containing everything except DNA were included in the PCR experiment. Five  $\mu\text{l}$  of each PCR product, including the controls, were ran on a 1% agarose gel to ensure that the expected 323 bp product was generated.

Restriction digest for the DNA fragment was carried out using Nla III restriction enzyme. Fifteen  $\mu$ l of the PCR product was digested for 16 h overnight at 37 C with 5 units of Nla III (New England Biolabs). The product of the restriction digest was mixed with 10  $\mu$ l of orange loading dye and ran on a 3% agarose gel (with Ethidium bromide) electrophoresis in a 1X Tris-Borate-EDTA buffer at 200V for 60 min. Although the presence of a G at position 1947 (COMT-codon 158) generated a unique 185 bp and 138 bp fragments, the 138 bp fragment was divided into unique 96 bp and 42 bp fragments when position 1947 contains an A as shown in Figure 3-1. The gels were visualized by UV and the RFLP gel electrophoresis products were read by two independent persons who were unaware of the identities of samples as either cases or controls.

#### **3.3.4. Statistical Analysis**

Statistical analysis was carried out using the Statistical Analysis System (SAS) software (Version 8.0). Conditional logistic regression was used to assess the association between the COMT genotypes and breast cancer risk. First all women were considered together in the analysis irrespective of menopausal status. Because there is evidence suggesting an interaction between some putative risk factors for breast cancer such as menopausal status and obesity, stratified analysis in premenopausal and postmenopausal women were carried out. Risk factors that were identified as significant predictors of breast cancer risk were controlled for in the final conditional logistic regression model involving all women. Further analyses controlling for these risk factors in stratified analysis based on menopausal status could not be carried out as some of the cells contain zero subjects.



### **3.4. RESULTS**

#### **3.4.1. Demographic characteristics**

Participants in this study consisted of 250 women with breast cancer and 250 age- and institution-matched controls drawn from four University Teaching Hospitals in Midwestern and Southeastern Nigeria. The mean age of the cases and controls were 46.1 years and 47.1 years respectively. The risk factors identified for breast cancer in this population include family history of breast cancer in first- and second-degree relatives (OR = 14.99, 95% CI 1.98-113.47), waist/hip ratio greater than the median in the controls (OR = 2.00, 95% CI 1.39-2.87), history of abortion (OR = 2.43, 95% CI 1.01-5.85), older age at first full term pregnancy (OR = 1.39, 95% CI 1.11-1.73) and higher level of education (High School and above) (OR = 1.31, 95% CI 1.07-1.61) as shown in Table 3-1. High parity (OR = 0.88, 95% CI 0.81-0.96) and longer duration of breastfeeding (OR = 0.75, 95% CI 0.62-0.91) conferred protection against breast cancer in these women.

Of the 250 breast cancer cases and 250 control subjects, PCR-based RFLP assays employing Nla III restriction enzyme were successful in 231 cases and 229 controls respectively. Figure 3-1 illustrates the results of the PCR-based RFLP assay for the COMT polymorphism on eight representative samples. It shows that the COMT (Val/Val), COMT (Val/Met), and COMT (Met/Met) are easily distinguishable using this technique.

### **3.4.2. Allele and genotype frequencies**

#### **All women**

As shown in Table 3-2, the frequencies of the COMT (Val) and COMT (Met) alleles among cases were 0.76 and 0.24 respectively and 0.73 and 0.27 respectively for control subjects for the entire sample. The frequencies of the COMT (Val) allele in the cases was slightly higher compared to the controls while the frequency of the COMT (Met) allele among the control subjects was slightly higher than in the cases but these were not significantly different.

As shown in Table 3-2, the COMT genotype frequencies were also not significantly different from between the cases and control subjects. The frequencies of the COMT (Val/Val), COMT (Val/Met), and COMT (Met/Met) in the cases 0.58, 0.38, and 0.05 respectively while the frequencies of these genotypes in the control subjects were 0.53, 0.40, and 0.07 respectively.

#### **Premenopausal women**

The frequency of the COMT (Val) allele in the cases (0.77) was slightly higher than that in the controls (0.72). Similarly, the frequency of the COMT (Met) allele was slightly lower in the cases (0.23) compared to the control subjects (0.28). There were slight differences in the distribution of the COMT genotypes in premenopausal women. The COMT (Val/Val) genotype was slightly higher in the control participants (0.59) compared to the cases (0.52). Conversely, the COMT (Val/Met) and COMT (Met/Met) genotypes were slightly lower in the cases compared to the controls as shown in Table 3-2.

### **Postmenopausal women**

The COMT (Val) allele was slightly higher in the cases (0.76) compared to the controls (0.74) while the COMT (Met) allele frequency was higher in the controls (0.27) compared to the cases (0.24). The distribution of the COMT (Val/Val), COMT (Val/Met), and COMT (Met/Met) genotypes were similar in the cases and controls. The frequencies of the COMT (Val/Val), COMT (Val/Met), and COMT (Met/Met) genotypes in the cases were 0.55, 0.41, and 0.04 respectively while the frequencies of these genotypes in the controls were 0.54, 0.39, and 0.07, respectively.

#### **3.4.3. COMT genotypes and breast cancer risk**

The strength of the association between the COMT genotypes and breast cancer risk was assessed using conditional logistic regression. First all women were considered together followed by subgroup analysis based on menopausal status.

### **All women**

Table 3-3 shows the results of the conditional logistic regression analysis. Slightly more cases [133 (57.88%)] than controls [121 (52.84%)] were homozygous for the high-activity COMT (Val/Val) genotype while fewer cases [87 (37.66%)] than controls [91 (39.74%)] carried the heterozygous intermediate-activity COMT (Val/Met) genotype. Carrying the heterozygous intermediate-activity COMT (Val/Met) genotype was associated with a reduced risk of breast cancer although this was not statistically significant (OR = 0.70, 95% CI 0.45-1.10). Also fewer cases [11 (4.76%)] than controls [17 (7.42%)] were homozygous for the low-activity COMT (Met/Met) genotype. When the intermediate-activity COMT (Val/Met) and low-activity COMT

(Met/Met) genotypes were combined in the logistic regression model, carrying at least one low-activity COMT (Met) allele was associated with a 26% reduced risk of breast cancer although this was not statistically significant (OR = 0.70, 95% CI 0.45-1.10).

Because it has been shown that some of the known risk factors for breast cancer such as obesity can modulate the effect of COMT genotypes on breast cancer risk, a model controlling for significant risk factors for breast cancer that were identified in the descriptive analysis was developed. In the final conditional logistic regression model, carrying at least one low-activity COMT (Met) allele was associated with a significant 43% reduced risk of breast cancer (OR = 0.57, 95% CI 0.36-0.91) as shown in Table 3-3.

### **Premenopausal women**

Of the 142 premenopausal breast cancer cases and 142 premenopausal controls, the PCR-based RFLP assay was successful in 128 cases and 128 controls. More cases [75 (58.59%)] than controls [67 (52.34%)] were homozygous for the high-activity COMT (Val/Val) genotype. Slightly fewer cases [46 (35.94%)] than controls [51 (39.84%)] carried the heterozygous intermediate-activity COMT (Val/Met) genotype. Heterozygosity was associated with a 33% reduced risk of premenopausal breast cancer (OR = 0.67, 95% CI 0.37-1.21) although this was not statistically significant. Slightly fewer cases [7 (5.47%)] than controls [10 (7.81%)] were homozygous for the low-activity COMT (Met/Met) genotype. There was an 11% non-significant reduced risk of premenopausal breast cancer associated with this genotype (OR = 0.89, 95% CI 0.46-1.73). A model combining the heterozygous intermediate-activity COMT (Val/Met) and the homozygous low-activity COMT (Met/Met) genotypes conferred a non-significant 21% reduced risk of premenopausal breast cancer (OR = 0.69, 95% CI 0.40-1.18). Adjustment for descriptive

risk factors in the conditional logistic regression model could not be carried out in subgroup analysis, as some of the cells contained zero subjects, thereby creating instability in the multivariate model.

### **Postmenopausal women**

Slightly more cases [57 (55.34%)] than control subjects [54 (54.00%)] were homozygous for the high-activity COMT (Val/Val) genotype. Also slightly fewer cases [42 (40.78%)] than controls [39 (39.00%)] carried the intermediate-activity COMT (Val/Met) genotype. There was a 17% reduced risk of postmenopausal breast cancer associated with carrying this genotype but this did not attain statistical significance (OR = 0.83, 95% CI 0.42-1.65). Although fewer cases [4 (3.88%)] than controls [7 (7.00%)] were homozygous for the low-activity COMT (Met/Met) genotype, this was not associated with risk of postmenopausal breast cancer (OR = 1.00, 95% CI 0.45-2.23). Combining individuals carrying the intermediate-activity COMT (Val/Met) and low-activity COMT (Met/Met) genotypes as a group in the logistic regression model conferred a non-significant 14% reduced risk of postmenopausal breast cancer (OR = 0.86, 95% CI 0.46-1.61).

### 3.5. DISCUSSION

This case-control study was designed to evaluate the association between the codon 158 G to A transition polymorphism in the COMT gene and breast cancer risk in Nigerian women, a population of exclusively African women. The frequency of 7.4% for the COMT-LL (Met/Met) genotype in the Nigerian control subjects in this study is 3-4 times lower than the 22-28% reported in US Caucasians<sup>20,21,23,29</sup> and half the 13% in African Americans<sup>23</sup> but close to the 9.8% found among Korean women<sup>24</sup> and 2 times higher than the 3.2% reported in Taiwanese control subjects<sup>25</sup>. The frequency of the COMT-HH (Val/Val) genotype among our control subjects (52.8%) is higher than corresponding figures in US Caucasians (22-36%)<sup>20,21,23,29</sup>, African Americans (42%)<sup>23</sup> but lower than the figures in Koreans (62%)<sup>24</sup> but similar to the frequency of this genotype among the Taiwanese (53%)<sup>25</sup>.

In the final logistic regression model controlling for family history of breast cancer, waist/hip ratio, age at first full-term pregnancy, and education, we found a significant reduced risk of breast cancer for carriers of at least one COMT-L (Met) allele (OR = 0.56, 95% CI 0.34-0.92) and a non-significant 13% reduced risk for homozygous carriers. This reduced risk for carrying at least one low activity COMT (Met) allele of the COMT gene was also noted in subgroup analysis based on menopausal status (31% in premenopausal women and 14% in postmenopausal women) although these were not statistically significant. This is in agreement with reports of some investigators<sup>20-22</sup> but in agreement with that of others<sup>21-25</sup>. In the Carolina Breast Cancer Study recruiting both Caucasians (389 cases and 379 controls) and African-

American women (265 cases and 263 controls), Millikan et al.<sup>20</sup> were unable to demonstrate any significant association between COMT (Val/Met) polymorphism and breast cancer risk overall or in subgroup analysis. In particular, there was a non-significant 20% reduced risk of breast cancer in African American women harboring both the heterozygous COMT (Val/Met) genotype (adjusted RRs = 0.8, 95% CI 0.5-1.2) and homozygous COMT (Met/Met) genotype (adjusted RRs = 0.8, 95% CI 0.4-1.5) in a multivariate unconditional logistic regression model controlling for significant descriptive risk factors identified in their population. Risk for breast cancer was also not related to the COMT (Val/Met) polymorphism among Caucasian women in their study (adjusted RR = 0.8, 95% CI 0.6-1.1 for the COMT [Val/Met] genotype and adjusted RR = 0.7, 95% CI 0.5-1.1 for the COMT [Met/Met] genotype). These authors also did not observe a positive association for COMT genotype among women who were physically inactive (recently or at age 12), who reported use of HRT or OC or who smoked cigarettes. In fact, their observation that women who were physically inactive showed a slightly inverse relationship with breast cancer risk was contrary to their hypothesis that women with low physical activity and low COMT activity will be at increased risk of breast cancer.

Two studies among Caucasian populations have reported conflicting associations between the COMT codon 158 polymorphism and breast cancer risk particularly in subgroup analysis. Although Lavigne et al.<sup>21</sup> (nested case control study recruiting 113 cases and 113 controls) noted no significant overall relationship between the COMT (Val/Met) polymorphism and breast cancer risk (overall associations with heterozygosity and homozygosity for the COMT (Met) allele were 1.30, 95% CI, 0.66-2.58 and 1.45, 95% CI, 0.69-2.58, respectively), they however reported that postmenopausal women homozygous for the COMT (Met) allele had a greater than two-fold increase in risk (OR = 2.18, 95% CI 0.93-5.11). Further stratified analysis

in postmenopausal women based on body mass index (BMI) showed significant associations only among those whose BMI was greater than 24.47 kg/m<sup>2</sup> (OR = 3.58, 95% CI 1.07-11.98). An inverse association between COMT (Val/Met) polymorphism was however noted in premenopausal women in their study (OR = 0.57, 95% CI 0.14-2.40 for the COMT [Val/Met] genotype and OR = 0.24, 95% CI 0.04-1.51 for the COMT [Met/Met] genotype).

On the other hand, in a case control study of 281 case patients and 289 control subjects in western New York, Thompson et al.<sup>22</sup> found that among premenopausal women with breast cancer, those with at least one low-activity allele showed significantly increased risk (OR=2.4; 95% CI, 1.4-4.3) and the risk was stronger on further stratification based on BMI (OR=5.7; 95% CI, 1.1-30.1 for the heaviest premenopausal women. There was an inverse association between low-activity alleles and postmenopausal breast cancer, which was most pronounced among those who were COMT (Met/Met) (OR=0.40; 95% CI, 0.2-0.7); and this risk was strongest on further stratification based on body mass index (BMI) in the leanest women with at least one low-activity allele (OR=0.3; 95% CI, 0.1-0.7). When COMT (Met/Val) individuals were combined with individuals who were COMT (Met/Met), having one or two low-activity alleles significantly decreased risk (OR=0.5; 95% CI, 0.3-0.9). Because the catechol estrogens are products of estrogen metabolism by CYP1A1 and CYP1A2, which are both induced by smoking, Ambrosone et al.<sup>23</sup> have also presented data evaluating the role of COMT on breast cancer in smoking and nonsmoking women. It is interesting that increased risk was observed only among postmenopausal women who smoked and that inverse associations were significant only among postmenopausal nonsmokers.

The results of the two studies among the Asian populations appear to demonstrate a more consistent association between the COMT polymorphism and breast cancer risk. Among Korean



women, Yim et al.<sup>24</sup> showed that in the total study population, subjects with at least one COMT-L (Met) allele had an almost two-fold risk of breast cancer compared with the COMT-HH (Val/Val) genotype individuals (OR=1.7; 95% CI=1.04-2.78). Categorization by menopausal status revealed no difference in the distribution of COMT genotypes between the different menopausal groups. In a multigenic study on the combined impact of CYP17, CYP1A1, and COMT on breast cancer risk among Taiwanese women, Huang et al.<sup>25</sup> compared the risk associated with harboring the high risk variants of these three genes in a case-control study recruiting 150 breast cancer cases and 150 hospital-based controls. The homozygous variants of the three polymorphisms evaluated (CYP17 A<sub>2</sub>/A<sub>2</sub>, CYP1A1 MspI vt/vt, and COMT L/L) were designated as the high-risk variants based on findings in their study. Individually, breast cancer risk associated with the susceptibility genotypes varied for the three genes and was much higher for COMT (P<0.05) than for CYP17 (P>0.05), and intermediate value for CYP1A1 (P<0.05). To determine whether the combined profiles of these estrogen-metabolizing genes may be associated with breast cancer, they examined breast cancer risk associated with combinations of these high-risk genotypes using women with the three putative low-risk genotypes as the reference groups. The authors found that the presence of at least one putative high-risk genotype was associated with increased risk of breast cancer. The risk of breast cancer increased significantly as the number of putative high-risk genotypes increased (p=0.006, based on Mantel extension test for a linear trend). Notably, none of the controls harbored all three high-risk genotypes. When genotype data was combined with other risk factors for breast cancer in the population for further analyses, it was discovered that harboring a high-risk COMT genotype is a stronger predictor of breast cancer risk than harboring a high-risk CYP17 or CYP1A1,

suggesting that in Taiwanese women inactivation of catechol estrogens may be more important than their formation in breast cancer development.

Overall, it is obvious that marked differences exist in the association of COMT genotypes and breast cancer risk even among studies in the same population. Some of these differences may be due to small sample size in some of the studies (Lavigne et al.<sup>20</sup>, 113 cases and 113 controls; Yim et al.<sup>24</sup>, 163 cases and 163 controls; and Huang et al.<sup>25</sup>, 150 cases and 150 controls). Although, our study sample size of 231 cases and 229 controls has 90% power to detect significant relationships between the COMT polymorphism and breast cancer risk in our study population, this was calculated based on the distribution of COMT genotypes in Caucasian populations. As already noted, the frequency of homozygous COMT (Val/Val), COMT (Val/Met), and COMT (Met/Met) genotypes in Caucasian controls (27%, 48%, and 25% respectively) differs significantly from the distribution of these genotypes in our control subjects (52.84%, 39.74%, and 7.42% respectively). Based on the above distribution, we speculate that a higher sample size will be required for detection of effects of COMT heterozygosity and homozygosity on breast cancer risk in our population compared to the Caucasian population. This is often one of the setbacks in early studies in populations with previously unknown genotype distributions. In addition, differences in statistical analysis may also contribute to difficulties in comparing results of various studies. Although all the above-cited studies were matched on age, only Lavigne et al.<sup>20</sup> performed conditional logistic regression. It is known that ORs may be overestimated when matched studies are analyzed with unconditional logistic regression. The effect of genotypes such as COMT on breast cancer risk may vary from one population to the other as a result of marked differences in the distribution of heterozygosity and homozygosity of the genotypes in the populations. The finding of interaction between COMT

genotypes and menopausal status and obesity may also partly explain the differences in reports from various populations. In the developed countries with a much higher percentage of postmenopausal breast cancer and a higher proportion of obese women, COMT may be expected to have more impact on risk of the disease compared to the Nigerian population with a smaller percentage of postmenopausal women in a predominantly lean population. It should be noted that for a genotype such as COMT with a very high frequency of heterozygosity and homozygosity in various populations, a strong positive link to breast cancer risk would have created an evolutionary disadvantage for humans.

About 70% of breast cancer patients in our study presented with advanced breast cancer (Manchester Stages III and IV). It is possible that we missed individuals with rapidly progressive breast cancer who may have died without reaching hospital. There is also no breast cancer-screening program in Nigeria. Therefore patients with early preclinical disease may have been missed in our study. We do not know how this would have influenced our results. Progress in molecular cancer genetics is occurring at a very rapid rate. It is expected that new procedures utilizing rapidly advancing high throughput techniques will soon replace studies based on single nucleotide polymorphisms (SNPs) in molecular cancer research. It is hoped that future molecular epidemiology cancer research in the next few decades will clarify our understanding of the contribution of low penetrance genes in the genesis of breast and other cancers.



**Figure 3-1: COMT NlaIII Restriction Digest**

**Table 3-1: Conditional logistic regression comparing cases and controls. Significant predictors of breast cancer risk [Numbers (N), Percentages (%)], [Means (S.D.)], age-adjusted odds ratio, 95% confidence interval**

Variable		Cases	Control	Odds ratio	95% CI
Family history of breast cancer	Yes	15 (6.00) <sup>a</sup>	1 (4.00) <sup>a</sup>	14.99	1.98,113.47
	No	235 (94.00) <sup>a</sup>	249 (96.00) <sup>a</sup>	1.00	
Waist/hip ratio (>0.90)	Yes	161 (64.40) <sup>a</sup>	117 (46.80) <sup>a</sup>	2.00	1.39,2.87
	No	89 (35.60) <sup>a</sup>	133 (53.20) <sup>a</sup>	1.00	
Abortion	Yes	18 (7.20) <sup>a</sup>	7 (2.80) <sup>a</sup>	2.43	1.01,5.85
	No	232 (92.80) <sup>a</sup>	243 (97.20) <sup>a</sup>	1.00	
Age at first full-term pregnancy		23.18 (5.36) <sup>b</sup>	21.87 (4.74) <sup>b</sup>	1.39	1.11,1.73
Education >= High School	Yes	136 (54.40) <sup>a</sup>	113 (45.20) <sup>a</sup>	1.31	1.07,1.61
	No	114 (54.60) <sup>a</sup>	137 (54.80) <sup>a</sup>	1.00	
Parity		4.1 (2.9) <sup>b</sup>	4.7 (3.1) <sup>b</sup>	0.88	0.81,0.96
Duration of breastfeeding		65.48 (47.32) <sup>b</sup>	80.96 (53.67) <sup>b</sup>	0.75	0.62,0.91

**Table 3-2: COMT allele and genotype frequencies for breast cancer cases and controls**

	Cases (n = 231)	Controls (n = 229)
All women		
Allele frequencies		
COMT-H (Val)	0.76	0.73
COMT-L (Met)	0.24	0.27
Genotype frequencies		
COMT-HH (Val/Val)	0.58	0.53
COMT-HL (Val/Met)	0.38	0.40
COMT-LL (Met/Met)	0.05	0.07
Premenopausal women		
Allele frequencies		
COMT-H (Val)	0.77	0.72
COMT-L (Met)	0.23	0.28
Genotype frequencies		
COMT-HH (Val/Val)	0.59	0.52
COMT-HL (Val/Met)	0.36	0.40
COMT-LL (Met/Met)	0.05	0.08
Postmenopausal women		
Allele frequencies		
COMT-H (Val)	0.76	0.74
COMT-L (Met)	0.24	0.27
Genotype frequencies		
COMT-HH (Val/Val)	0.55	0.54
COMT-HL (Val/Met)	0.41	0.39
COMT-LL (Met/Met)	0.04	0.07

**Table 3-3: Distribution of polymorphisms of the COMT gene in relation to breast cancer**

	Cases (unmatched)	Controls (unmatched)	OR (95% CI) (matched)	OR (95% CI) <sup>a</sup> (matched)
All women				
COMT-HH (Val/Val)	133 (57.58)	121 (52.84)	1.00	1.00
COMT-HL (Val/Met)	87 (37.66)	91 (39.74)	0.70 (0.45-1.10)	0.56 (0.34-0.92)
COMT-LL (Met/Met)	11 (4.76)	17 (7.42)	1.00 (0.59-1.69)	0.87 (0.47-1.59)
COMT-HH (Val/Val)	133 (57.58)	121 (52.84)	1.00	
COMT-HL+LL (Val/Met+Met/Met)	98 (42.42)	108 (47.16)	0.74 (0.49-1.12)	0.57 (0.36-0.91)
Pre-menopausal women				
COMT-HH (Val/Val)	75 (58.59)	67 (52.34)	1.00	
COMT-HL (Val/Met)	46 (35.94)	51 (39.84)	0.67 (0.37-1.21)	
COMT-LL (Met/Met)	7 (5.47)	10 (7.81)	0.89 (0.46-1.73)	
COMT-HH (Val/Val)	75 (58.59)	67 (52.34)	1.00	
COMT-HL+LL (Val/Met+Met/Met)	53 (41.51)	61 (47.65)	0.69 (0.40-1.18)	
Postmenopausal women				
COMT-HH (Val/Val)	57 (55.34)	54 (54.00)	1.00	
COMT-HL (Val/Met)	42 (40.78)	39 (39.00)	0.83 (0.42-1.65)	
COMT-LL (Met/Met)	4 (3.88)	7 (7.00)	1.0 (0.45-2.23)	
COMT-HH (Val/Val)	57 (55.34)	54 (54.00)	1.00	
COMT-HL+LL (Val/Met+Met/Met)	46 (44.66)	46 (46.00)	0.86 (0.46-1.61)	

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#### **4. CYTOCHROME P4501A1 GENETIC POLYMORPHISMS AND BREAST CANCER RISK IN NIGERIAN WOMEN**

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#### 4.1. ABSTRACT

In this case-control study based on 250 women with breast cancer and 250 age-matched controls, we sought to evaluate the role of four polymorphic variants in the CYP1A1 gene in breast cancer susceptibility in Nigerian women. Heterozygosity for the CYP1A1 M1 genotype (CYP1A1 M1 [T/C]) was associated with a 21% reduced risk of breast cancer (OR = 0.79, 95% CI 0.46-1.40) while homozygosity for the genotype (CYP1A1 M1 [C/C]) conferred a non-significant 9% reduced risk of breast cancer. These risk profiles were not significantly altered in subgroup analysis by menopausal status. While heterozygosity for the CYP1A1 M3 genotype (T/C) conferred a non-significant 24% reduced risk of breast cancer (OR = 0.76, 95% CI 0.47-1.22), homozygosity for the variant was associated a non-significant 1.95-fold increased risk of breast cancer (OR = 1.95, 95% CI 0.24-6.01). Subgroup analysis showed a non-significant 11% reduced risk in premenopausal heterozygous carriers (OR = 0.89, 95% CI 0.45-1.44) and a non-significant 6% increased risk of postmenopausal breast cancers for carriers of the CYP1A1 M3 (T/C) genotype. The CYP1A1 M2 (isoleucine to valine) polymorphism in exon 7 and CYP1A1 M4 (threonine to asparagine) variant in codon 461 of the CYP1A1 gene were found to be very rare in our study subjects. This study has shown that while the CYP1A1 M1 polymorphism conferred reduced risk of breast cancer, homozygosity for the CYP1A1 M3 (C/C) was associated with increased risk of breast cancer although these risks did not attain statistical significance.

## 4.2. INTRODUCTION

Cytochrome P450 1A1 (CYP1A1) gene plays a central role in the 2-hydroxylation of estradiol and estrone (the two estrogens in humans) to 2-hydroxy catechol metabolites for subsequent O-methylation to 2-methoxy intermediates. While the 2-hydroxylation products (2-OH estradiol catechol and 2-OH estrone catechol) are devoid of estrogenic activities<sup>1</sup> and the 2-methoxy derivatives shown to possess anti-proliferative and anti-angiogenic properties<sup>2-4</sup>, another mutually exclusive pathway of 16 $\alpha$ -hydroxylation leads to metabolites with strong estrogenic properties and have been linked to estrogen-induced carcinogenesis in both laboratory animals and humans<sup>5-7</sup>.

The CYP1A1 gene is highly polymorphic in human populations and ethnic differences in the distribution of these polymorphisms have been reported in various populations. Four polymorphisms including M1 (a threonine to cysteine substitution in the 3' non-coding region)<sup>8</sup>, M2 (isoleucine to valine variant in codon 462 in the heme-binding domain in exon 7)<sup>9</sup>, M3 (an A-T to G-C transition mutation in the 3' non-coding region 300 base pairs from the polyadenylation site)<sup>10</sup>, and M4 (a threonine to asparagine substitution in codon 461)<sup>11</sup> have been described in various populations. It has been demonstrated by Crofts et al.<sup>12</sup> that the exon 7 polymorphism has a role in gene function by increasing both enzyme activity and mRNA levels in Asians. The same investigators found an increase in CYP1A1 mRNA in Caucasians who carry the double heterozygous genotype for the CYP1A1 M1 and CYP1A1 M2 (exon 7) polymorphisms, but no evidence for a functional significance of the exon 7 polymorphism alone. Association of CYP1A1 polymorphisms and breast cancer risk has been reported in various

populations with inconsistencies even within populations. Taoli et al.<sup>13,14</sup> observed an increased risk of breast cancer among a small sample of African American women (20 cases and 81 controls) but not among Caucasian women who harbor the CYP1A1 M1 variant. Rebbeck et al.<sup>15</sup> found no association between the exon 7 polymorphism (M2) and breast cancer risk among US Caucasian women (96 cases and 146 controls) while Ambrosone and colleagues<sup>16</sup> noted an increased risk of postmenopausal breast cancer among Caucasian women with the variant exon 7 allele (216 cases and 282 controls). While Ishibe et al.<sup>17</sup> reported that neither the CYP1A1 M1 nor CYP1A1 M2 polymorphisms was independently associated with overall breast cancer risk, these investigators noted an interaction between these variants and smoking; current smoking and adolescent-onset smoking interacted with CYP1A1 M1 and CYP1A1 M2, respectively to increase breast cancer risk among Caucasian women in a case-control study nested in the Nurses' Health Study. Studies in Asian populations have also reported conflicting results. While Huang et al.<sup>18</sup> noted an increased risk among homozygous carriers of the CYP1A1 M1 polymorphism among postmenopausal Chinese women in Taiwan, Miyoshi and colleagues<sup>19</sup> reported a significant 40% and 34% reduced risk of breast cancer for the CYP1A1 M1 and CYP1A1 M2 variants in Japanese women.

Apart from the small data from Taoli et al.<sup>13,14</sup> noted above, we are unaware of any other association study of CYP1A1 genetic polymorphisms and breast cancer risk in women of African descent in the literature. Because of the wide ethnic differences in the distribution of CYP1A1 gene polymorphisms, this study recruiting 250 women with breast cancer and 250 age-matched control subjects seeks to evaluate the association between CYP1A1 M1, M2, M3, and M4 polymorphisms and breast cancer risk in an exclusively African population drawn from Midwestern and Southeastern Nigeria.

### **4.3. MATERIALS AND METHODS**

#### **4.3.1. Recruitment of study participants**

Two hundred and fifty cases of breast cancer and 250 age-matched women without the disease were recruited from four University Teaching Hospitals located in Midwestern and Southeastern Nigeria between September 2002 and April 2004 for this case-control study. Study protocols were approved by the Ethics and Research Committees of the Nigerian institutions and the Institutional Review Board (IRB) of the University of Pittsburgh. The cases were women with breast cancer being managed at the surgical out-patient clinics and surgical wards of the Nigerian hospitals. Control subjects were women without evidence of breast and other malignant diseases who were being treated for other surgical disorders including road traffic accidents, intestinal obstructions, chronic gallbladder diseases, appendicitis and non-malignant leg ulcers.

Eligible study participants were identified by with the assistance of physicians in these hospitals who informed them about the study and referred those willing to the study investigators. Details of the study including objectives, risks and benefits, confidentiality and right of participation were explained to eligible participants. Potential study participants were free to discuss participation with close family members. Written informed consent was obtained those that accepted to participate in the study.

Interviewer-administered questionnaires detailing sociodemographic characteristics including age, sex, occupation, exposure to chemical fertilizers and pesticides and obstetric and gynecological variables such as age at menarche, parity, age at first full-term pregnancy (FFTP), age at menopause, use of hormone contraceptives and hormone replacement therapy and history



of abortions and surgical oophorectomy were used for data collection, which lasted 30 minutes. Height, weight, waist and hip circumferences were measured at the end of the interview.

#### **4.3.2. Biological samples**

Forty milliliters of whole blood collected in two 15 ml plain vacutainer tubes and one 10 ml K<sub>3</sub>-EDTA vacutainer tube was obtained from each study participant. Samples were centrifuged within 10 h of collection; buffy coat separated from plasma and red cells in the K<sub>3</sub>-EDTA vacutainer tubes were carefully pipetted into 3 ml tubes while clots separated from serum in the plain vacutainer tubes were turned into two 20 plain tubes and stored at –20 °C in each of the Nigerian study sites until transferred in polar ice packs to the Nigerian coordinating center at the University of Benin Teaching Hospital where samples were stored at –20 °C. The samples were later transferred to the University of Pittsburgh in dry ice and stored at –80 °C until DNA extraction.

#### **4.3.3. DNA extraction**

DNA extraction was from buffy coats and blood clots for participants in whom buffy coat was unavailable. QIAamp DNA Mini Kit<sup>20</sup> and QIAamp DNA Midi Kit<sup>21</sup> protocols were used for DNA extraction from buffy coats and blood clots respectively. The DNA was stored at 4 °C until amplified by polymerase chain reaction (PCR) and used for restriction fragment length polymorphism (RFLP) analysis.

#### 4.3.4. PCR and Genotyping

Polymerase chain reaction (PCR) amplification and restriction endonuclease digest were carried out for each of the four CYP1A1 variants (CYP1A1 M1, M2, M3 and M4). Primers used for the PCR reaction, fragment lengths amplified and restriction enzymes used are detailed in Table 4-1. For the CYP1A1 M1 variant, a 348 base pair (bp) fragment containing part of exon 7 and the adjoining 3' non-coding region of intron 7 was amplified using the following primers (Forward [C1]: 5' CCG CTG CAC TTA AGC AGT CT 3', Reverse [C2]: 5'AGG GCG TAA GTC AGC ACA GT 3'. For the CYP1A1 M2 and CYP1A1 M4 polymorphisms, a 377 bp fragment containing part of exon 7 bearing the M2 and M4 variants was amplified using the following primers (Forward [C3]: 5' GCA TTG ATC CTC CTG TCC AT, Reverse [C4]: 5' AGG CAT GCT TCA TGG TTA GC 3'). A 400 bp fragment encompassing part of 3' non-coding region of the gene was amplified for the CYP1A1 M3 polymorphism using the following primers (Forward [C5]: 5' GGC CTC TGA GAA GCT CTG AA 3', Reverse [C6]: 5' GTC CTG GTG CCT GGA TAT GT 3').

For each of the four polymorphisms, a 50 µl PCR reaction mixture containing 2 µl of genomic DNA, 8 µl of deoxynucleotide triphosphates, 1 µl each of forward and reverse primers, 5 µl of 10X buffer, 1.5 µl of MgCl and 0.2 µl of Taq polymerase was placed in a MJ Research DYAD thermocycler. After denaturing for 5 min at 95 °C, the DNA was amplified for 35 cycles at 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s, followed by a 5 min extension at 72 °C. A positive control containing genomic DNA (University of Pittsburgh Molecular Epidemiology Laboratory control) and a negative control containing everything except DNA were included in

the PCR experiment. Five µl of each PCR product, including the controls, were run on a 1% agarose gel to ensure that the expected fragments product was generated.

Restriction digest for each of the four variants of the CYP1A1 gene was carried out using restriction endonucleases detailed in Table I: CYP1A1 M1, MspI; CYP1A1 M2, BsrDI; CYP1A1 M3, MspI; and CYP1A1 M4, BsaI. Digestion for the CYP1A1 M1 MspI variant carried out at 37 °C overnight for 16 h revealed a band of 348 bp for the CYP1A1 M1 (T) allele and two bands of 230 bp and 118 bp for the CYP1A1 M1 (C) allele (Fig 4-1). BsrDI digest for the CYP1A1 M2 polymorphism, carried out for 16 h overnight at 65 °C, revealed a 377 bp fragment for the G allele and two bands of 237 bp and 140 bp for the A allele as shown in Figure 4-2. For the CYP1A1 M3 variant, a 400 bp fragment for the T allele and two fragments of 330 bp and 70 bp for the C allele were detected following a 16 h digestion at 37 °C and ran on 3% agarose gel electrophoresis stained with ethidium bromide as shown in Figure 4-3. Digestion for the CYP1A1 M4 variant with BsaI restriction endonuclease overnight for 16 h at 50 °C yielded a 377 bp fragment for the A allele and two fragments of 277 bp and 150 bp for the C allele (Figure 4-4).

#### **4.3.5. Statistical Analysis**

Data analysis was carried out using Statistical Analysis Systems (SAS) software (Version 8.0). Assessment of association between socio-demographic characteristics and obstetric and gynecological variables and breast cancer risk was first carried out using conditional logistic regression. Because PCR amplification and RFLP analysis for the CYP1A1 M2 and M4 variants revealed that all individuals genotyped for CYP1A1 M2 variant carried the wild type CYP1A1 M2 (A) allele except for one individual with the heterozygous CYP1A1 (A/G) genotype, and all

the study subjects genotyped for the CYP1A1 M4 polymorphism were homozygous for the C allele (CYP1A1 M4 [C/C]), we restricted analysis of association between CYP1A1 genotypes and breast cancer risk to the CYP1A1 M1 and CYP1A1 M3 variants. Conditional logistic regression was used to evaluate association between these two CYP1A1 variants and breast cancer risk. First, all women were combined in univariate conditional logistic regression model restricted to each of the variants. Next variables that were found to be significant predictors of breast cancer risk in the descriptive analysis were entered into the multivariate conditional logistic regression models together with the genotype data. Subgroup analysis based on menopausal status was carried out separately for premenopausal and postmenopausal women.

## **4.4. RESULTS**

### **4.4.1. Descriptive epidemiology**

Results of conditional logistic regression models evaluating the association of various socio-demographic and obstetric and gynecological variables are shown in Table 4-2. Family history of breast cancer (OR = 14.99, 95% CI 1.98-113.47), waist/hip ratio (OR = 2.00, 95% CI 1.39-2.87), history of abortion (OR = 2.43, 95% CI 1.01-5.85), older age at first full-term pregnancy (OR = 1.39, 95% CI 1.11-5.85) and education above High School (OR = 1.31, 95% CI 1.07-1.61) were associated with increased risk of breast cancer. A 12% reduced risk of breast cancer was conferred by high parity (OR = 0.88, 95% CI 0.81-0.96); longer duration of breastfeeding was also associated with a significant 25% reduced risk of breast cancer (OR = 0.75, 95% CI 0.62-0.91).

#### **4.4.2. Association of CYP1A1 M1 polymorphism and breast cancer risk**

PCR-based RFLP assay for the CYP1A1 M1 polymorphism was successful in 220 cases and 218 control subjects out of 250 cases and 250 controls that were recruited for the study. The CYP1A1 M1 variant was found to be highly polymorphic in the study population as shown in Table 4-3.

##### **All women**

Allele frequencies for the CYP1A1 M1 (T) and CYP1A1 M1 (C) alleles were 0.77 and 0.23 respectively among the cases and 0.76 and 0.24 respectively among the control subjects. Frequencies for the CYP1A1 M1 (T/T), CYP1A1 M1 (T/C) and CYP1A1 M1 (C/C) genotypes for the cases (0.61, 0.31, and 0.07 respectively) were not significantly different from the distribution of these genotypes in the control subjects (0.60, 0.33, and 0.08, respectively). When compared with the homozygous CYP1A1 M1 (T/T) genotype carriers, individuals harboring the heterozygous CYP1A1 M1 (T/C) genotype had a non-significant 21% reduced risk of breast cancer (OR = 0.79, 95% CI 0.46-1.40) in the final multivariate conditional logistic regression model controlling for other identified risk factors for breast cancer as shown in Table 4-4. Homozygosity for the CYP1A1 M1 (C/C) allele also conferred a non-significant 11% reduced risk of breast cancer in the final model (OR = 0.91, 95% CI 0.50-1.66). Combining heterozygous CYP1A1 M1 (T/C) and CYP1A1 M1 (C/C) carriers was associated with a non-significant 21% reduced risk of breast cancer.

### **Premenopausal women**

The distribution of the CYP1A1 M1 (T) allele and CYP1A1 M1 (C) allele in premenopausal breast cancer cases was not significantly different from the frequency of these alleles in the control subjects. While the allele frequencies in the cases were 0.78 and 0.22 respectively for the T and C alleles, the frequencies of the T and C alleles in the control subjects were 0.75 and 0.25. Slightly more cases (78 [62.40%]) than controls (71 [57.26%]) were homozygous for CYP1A1 M1 (T/T) genotype while fewer cases (40 [32.00%]) than controls (43 [34.68%]) carried the heterozygous CYP1A1 M1 (T/C) genotype. Seven cases (5.60%) and 10 controls (8.06%) were homozygous for the CYP1A1 M1 (C) variant. There was a non-significant 38% reduced risk of premenopausal breast cancer among carriers of the heterozygous CYP1A1 M1 (T/C) genotype (OR = 0.62, 95% CI 0.31-1.24). Similarly, homozygosity for the CYP1A1 M1 (C) allele conferred a non-significant 37% reduced risk of premenopausal breast cancer (OR = 0.63, 95% CI 0.28-1.44). Although, there was a 42% reduced risk of premenopausal breast cancer when the CYP1A1 M1 (T/C) and CYP1A1 M1 (C/C) genotypes were pooled together, this was also not statistically significant (OR = 0.58, 95% CI 0.31-1.09).

### **Postmenopausal women**

Of the 108 cases of postmenopausal breast cancer patients and 108 age-matched controls, the PCR-based RFLP assay was successful in 103 cases and 104 control subjects. Slightly fewer cases (62 [60.19%]) than controls (58 [61.70%]) were homozygous for the CYP1A1 M1 (T) allele. Almost equal number of cases (31 [30.10%]) and controls (29 [30.85%]) harbored the heterozygous CYP1A1 M1 (T/C) genotype while slightly more cases (10 [9.71%]) than controls

(7 [7.45%]) were homozygous for the CYP1A1 M1 (C) allele. Heterozygosity for the CYP1A1 M1 variant (CYP1A1 M1 [T/C]) was not related to the risk of postmenopausal breast cancer (OR = 1.00, 95% CI 0.49-2.05) while homozygosity for the CYP1A1 M1 (C) allele (CYP1A1 M1 [C/C]) conferred a non-significant 16% increased risk of postmenopausal breast cancer (OR = 1.16, 95% CI 0.55-2.44). Combining individuals heterozygous for the CYP1A1 M1 genotype 1 [T/C]) and homozygous carriers of the variant (CYP1A1 M1 [C/C]) was associated with a non-significant 6% increased risk of postmenopausal breast cancer (OR = 1.06, 95% CI 0.55-2.01).

#### **4.4.3. Association of CYP1A1 M3 polymorphism and breast cancer risk**

##### **All women**

As shown in Table 4-5, the CYP1A1 M3 variant is highly polymorphic in the study population with allele frequencies of 0.88 and 0.12 for the CYP1A1 M3 (T) and CYP1A1 M3 (C) alleles respectively among the cases and 0.87 and 0.13 for the CYP1A1 M3 (T) and CYP1A1 M3 (C) alleles respectively among the control subjects. Slightly more cases (176 [76.86%]) than control subjects (172 [75.77%]) were homozygous for the CYP1A1 M3 (T) allele while almost equal number of cases (52 [22.71%]) and control subjects (53 [23.35%]) carried the heterozygous CYP1A1 M3 (T/C) genotype. Only one case (0.44%) and two controls (0.88%) harbored the homozygous CYP1A1 M3 (C/C) genotype. In the final multivariate conditional logistic regression model, heterozygosity for the CYP1A1 M3 genotype (CYP1A1 M3 [T/C]) conferred a non-significant 24% reduced risk of breast cancer (OR = 0.76, 95% CI 0.47-1.22) while homozygosity for the CYP1A1 M3 (C) allele (CYP1A1 M3 [C/C]) was associated with a non-significant 95% increased risk of breast cancer (OR = 1.95, 95% CI 0.24-6.01) as shown in Table 4-6.

### **Premenopausal women**

Of the 142 cases of premenopausal breast cancer and the 142 age-matched premenopausal controls, the PCR-based RFLP assay was successful in 126 cases and 126 controls. The distribution of the CYP1A1 M3 (T) and CYP1A1 M3 (C) alleles in the cases (0.87 and 0.13 respectively) is not significantly different from the distribution of these alleles in the control subjects (0.85 and 0.15, respectively). As shown in Table 4-5, slightly more cases (95 [75.40%]) than controls (90 [71.43%]) were homozygous for the CYP1A1 M3 (T) allele while fewer cases (30 [23.81%]) than controls (35 [27.78%]) carried the heterozygous CYP1A1 M3 (T/C) genotype. While heterozygosity was associated a non-significant 19% reduced risk of premenopausal breast cancer (OR = 0.81, 95% CI 0.45-1.44), homozygosity for the CYP1A1 M1 (C) allele was not related to premenopausal breast cancer risk (OR = 1.00, 95% CI .025-4.00). Combining heterozygous and homozygous carriers of the CYP1A1 M3 (C) allele did not alter the premenopausal breast cancer risk as shown in Table 4-6 (OR = 0.82, 95% CI 0.46-1.43).

### **Postmenopausal women**

The PCR-based RFLP assay was successful in 103 out of 108 postmenopausal breast cancer cases and 99 out of 108 postmenopausal control subjects. CYP1A1 M3 (T) allele and CYP1A1 M3 (C) allele frequencies were similar in cases (0.89 and 0.11, respectively) and control subjects (0.90 and 0.10, respectively) as shown in Table 4-5. Slightly fewer cases (81 [78.64%]) than controls (80 [80.81%]) were homozygous for the CYP1A1 M3 (T) allele while slightly more cases (22 [21.36%]) than controls (18 [18.18%]) carried the heterozygous CYP1A1 M3 (T/C) genotype as shown in Table 4-6. Compared with the homozygous CYP1A1 M3 (T/T)



carriers, heterozygosity (CYP1A1 M3 (T/C)) was associated with a non-significant 6% increased risk of postmenopausal breast cancer (OR = 1.06, 95% CI 0.54-1.20). Risk associated with the homozygous CYP1A1 M3 (C/C) genotype could not be assessed as none of the postmenopausal breast cancer cases carried the homozygous CYP1A1 M3 (C/C) genotype.

#### **4.5. DISCUSSION**

CYP1A1 may be involved in breast cancer via estrogen-related mechanisms. Estrogens are metabolized by two competing pathways to form either inactive 2-hydroxy-estradiol and estrone or active 16 $\alpha$ -hydroxy-estradiol and estrone, respectively; level of the latter are often elevated in breast cancer, and may be related to tumorigenesis<sup>22</sup>. CYP1A1 polymorphism may affect the distribution of these metabolites and thus determine susceptibility to cancer<sup>23,24</sup>. To date, four polymorphisms have been described within this gene; M1, M2, M3, and M4. It has been suggested that there is wide ethnic variation in the distribution of polymorphisms of the CYP1A1 gene. For example, the CYP1A1 M2 (Exon 7) polymorphism was found to be rare in our study population with only one heterozygous (CYP1A1 M2 [A/G]) carrier in the entire sample. This same polymorphism has been reported to have allele frequencies of 0.69 and 0.31 among the Japanese<sup>8</sup>, and 0.88 and 0.12 among a racially mixed African American population<sup>25</sup>. Studies in Asian populations indicate that more than 10% of the population possesses variant genotypes of this polymorphism<sup>18</sup>. Allele frequencies of this polymorphism in Caucasians have been reported to be low (0.97 and 0.03)<sup>15</sup>. Also we did not find any carriers of the CYP1A1 M4 (A) allele in our study subjects; all were homozygous for the CYP1A1 M4 (C) allele. On the other hand, the CYP1A1 M1 and CYP1A1 M3 variants were highly polymorphic among the

Nigeria study subjects. Allele frequencies for the CYP 1A1 M1 (T) and CYP1A1 (C) alleles among our control subjects were 0.76 and 0.24 respectively with genotype frequencies of 0.60, 0.33, and 0.08 for the CYP1A1 M1 (T/T), CYP1A1 M1 (T/C), and CYP1A1 M1 (C/C) genotypes respectively. The homozygous CYP1A1 M1 (C) frequency of 8% in this study agrees with the findings of Cosma et al.<sup>26</sup> These investigators reported the frequency of the CYP1A1 M1 (C/C) to be 6-7% in a small sample of African American women (21 cases and 85 controls). Our finding is however lower than the 13% reported for the homozygous CYP1A1 M1 (C/C) genotype among Asians but higher than the 2% reported in Caucasian populations<sup>26</sup>.

The CYP1A1 M3 polymorphism has been reported previously only in African American women. We have found this variant to also be polymorphic in Nigerian women occurring with allele frequencies of 0.87 and 0.13 for the CYP1A1 M3 (T) and CYP1A1 M3 (C) alleles and among the control subjects. Among a small sample of African American women, Crofts et al.<sup>27</sup> observed a 17% carrier rate for the CYP1A1 M3 variant with CYP1A1 M3 (T) and CYP1A1 M3 (C) allele frequencies of 0.91 and 0.09 respectively.

Breast cancer risk association studies with CYP1A1 variants in the literature have been conflicting. We did not observe any significant association between the two common CYP1A1 polymorphisms in our study population (CYP1A1 M1 and CYP1A1 M3) and breast cancer risk overall, or in subgroup analysis based on menopausal status. In particular, the CYP1A1 M1 polymorphism was associated with a non-significant 19% reduced risk of breast cancer (OR = 0.79, 95% CI 0.46-1.40). This same polymorphism was reported to confer almost 10-fold increased risk of breast cancer among African American women by Taioli et al.<sup>14</sup> (OR = 9.7, 95% CI 2.0-47.9) although their study was of small sample size (21 cases and 85 controls). These investigators were unable to demonstrate any association of CYP1A1 M1 polymorphism

and breast cancer risk in Caucasian women in their study (OR = 1.7, 95% CI 0.6-4.9 for heterozygous carriers of the CYP1A1 M1 [T/C] genotype).

Rebbeck et al.<sup>15</sup> observed no association of breast cancer risk with the exon 7 polymorphism among 96 incident breast cancer cases and 146 controls, whereas Ambrosone and colleagues<sup>16</sup> reported a significant increase in risk among postmenopausal women with the variant exon 7 allele (based on 216 cases and 282 controls). In addition, an interaction was observed in the latter study with smoking, suggesting that this polymorphism may be important in increasing breast cancer risk among light smokers (defined as <30 pack-years). In a prospective study of 466 cases (over 99% Caucasians) and an equal number of controls nested in the Nurses' Health Study, Ishibe et al.<sup>17</sup> were unable to detect an overall increase in breast cancer risk with the variant CYP1A1 genotypes. There was however a suggestion of a role of the variant alleles in breast cancer development in association with smoking. An increase in breast cancer risk among smokers was limited to those who were variant for the CYP1A1 M1 polymorphism and who had smoked greater than 29 pack-years contrary to the findings of Ambrosone et al.<sup>16</sup> noted above.

In a case-control study recruiting 150 postmenopausal Chinese women with breast cancer and an equal number of controls, Huang et al.<sup>18</sup> found that the homozygous variant of the CYP1A1-MspI polymorphism was a significant predictor of breast cancer risk, independent of other established risk factors (adjusted odds ratio 1.98; 95% CI, 1.01-3.99). In a recent study among Japanese women, Miyoshi et al.<sup>19</sup> found that carriers with variant allele 6235C (MspI polymorphism) or variant allele 462Val (exon 7) have significantly lower risk of breast cancer, that is, the ORs were 0.60 for the 6235C (MspI polymorphism) carriers and 0.66 for allele 462Val (exon 7) carriers compared with noncarriers. They also noted linkage disequilibrium

between the two variants because most of the variant 462Val carriers were associated with the variant allele 6235C (MspI polymorphism) carriers by haplotype analysis. These findings are inconsistent with those reported for other ethnicities; that is, carriers of these variant alleles have a breast cancer risk equivalent to noncarriers in Caucasian women<sup>13,17,28</sup> and carriers of variant allele 6235C (MspI) have a significantly higher breast cancer risk than noncarriers in African-American women<sup>13,14</sup> and similar to the findings in postmenopausal Chinese women in Taiwan<sup>18</sup>. The authors admitted that reasons for their finding are currently unknown but postulated that this may be partly explained by the differences in other genetic and environmental factors among these ethnicities

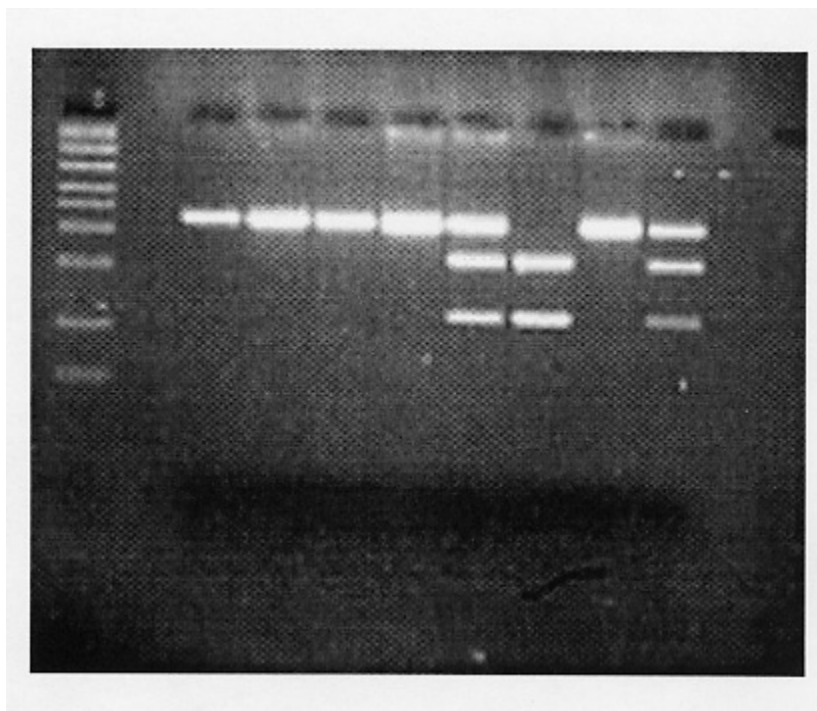
Some of the limitations of our study have to do with recall and other biases associated with case-control studies. However, the genetic constitution of individuals is constant and not susceptible to recall bias. Control subjects for our study were recruited from hospital patients; this group of control patients may not be exactly representative of the general population from which the cases were drawn. It should however be noted that with limited communication facilities in developing countries such as Nigeria, population controls are often difficult to recruit. Sample size may also be a factor of concern. Although we calculated sample size based on available data in the literature, some of the studies used were of small sample size and may not be very reliable for accurate sample size determination. Over 70% of breast cancer patients presented with advanced stages of the disease (Manchester Stages III and IV). It is possible that we may have missed patients with rapidly progressive breast cancer who may have died before reaching the stage of fungating tumors seen in most of our patients. There is currently no breast cancer-screening program in Nigeria; it is possible that we also missed patients with early stage

preclinical breast cancer. We do not know how these issues may have affected our genotype data.

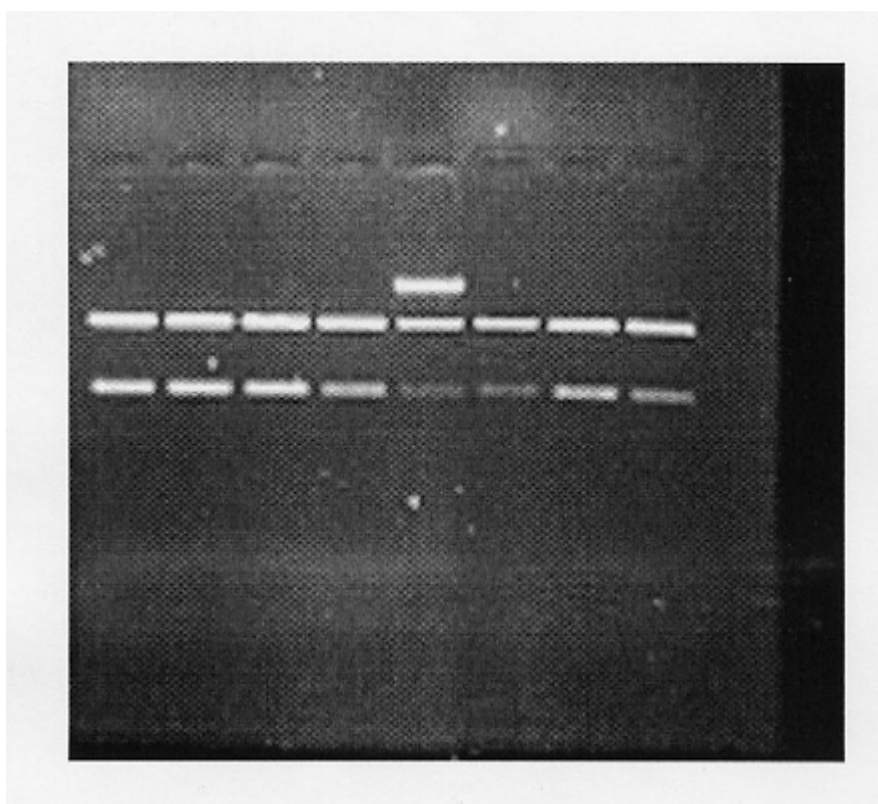
Although we were unable to demonstrate a statistically significant association between CYP1A1 polymorphisms and breast cancer risk, our findings are in keeping with some reports in the literature<sup>17,18</sup> and at variance with others<sup>14</sup>. It should be noted that the contribution of genetic variations to breast cancer risk may vary from one population to another on account of differences in the prevalence of various polymorphisms across populations and because our is the first study to report on the relationship between CYP1A1 polymorphisms and breast cancer risk in sub-Saharan African populations, more studies are suggested to further elucidate the contribution of CYP1A1 genetic variants to breast cancer risk in African populations. It is our hope that new techniques employing high through put microarray and proteonomic technologies may contribute to our understanding of the role of genetic variants to susceptibility to breast cancer and other malignancies in the next decades.

**Table 4-1: CYP1A1 Polymorphisms analysis by restriction enzyme digest**

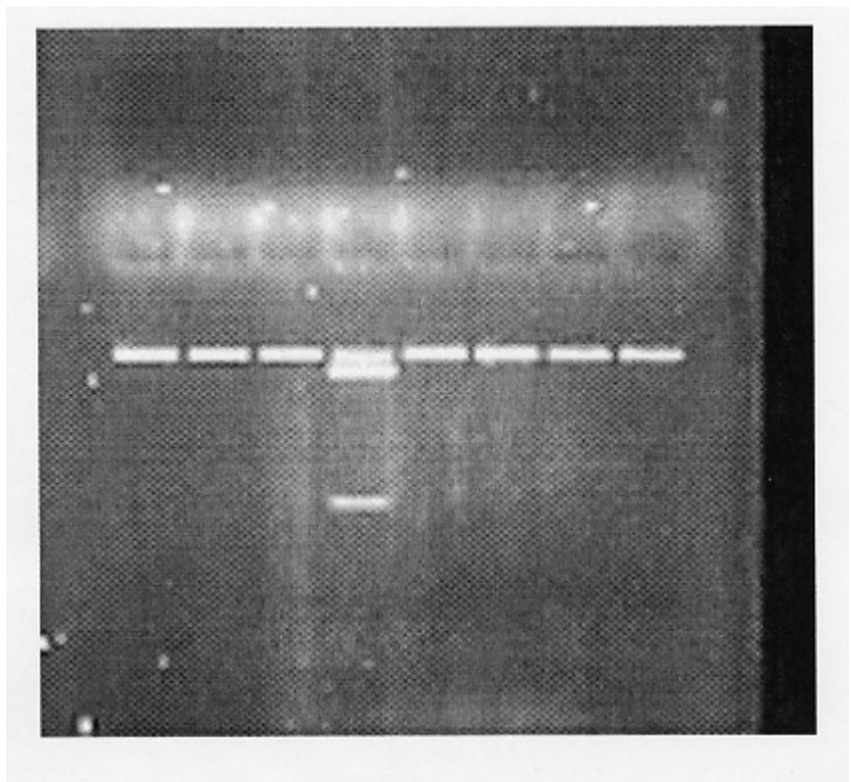
Polymorphism	Primers	Restriction enzyme	Fragment
M1: T6235C creates a new MspI site	C1, C2	MspI	T (348), C (230, 118)
M2: A4889G results in Ile462Val and may increase enzyme activity	C3, C4	BsrDI	G (377), A (237, 240)
M3: T5639C creates a new MspI site	C5, C6	MspI	T (400), C (330, 70)
C4887A results in Thr461Asn with unknown functional effect	C3, C4	BsaI	A (377), C (227, 150)



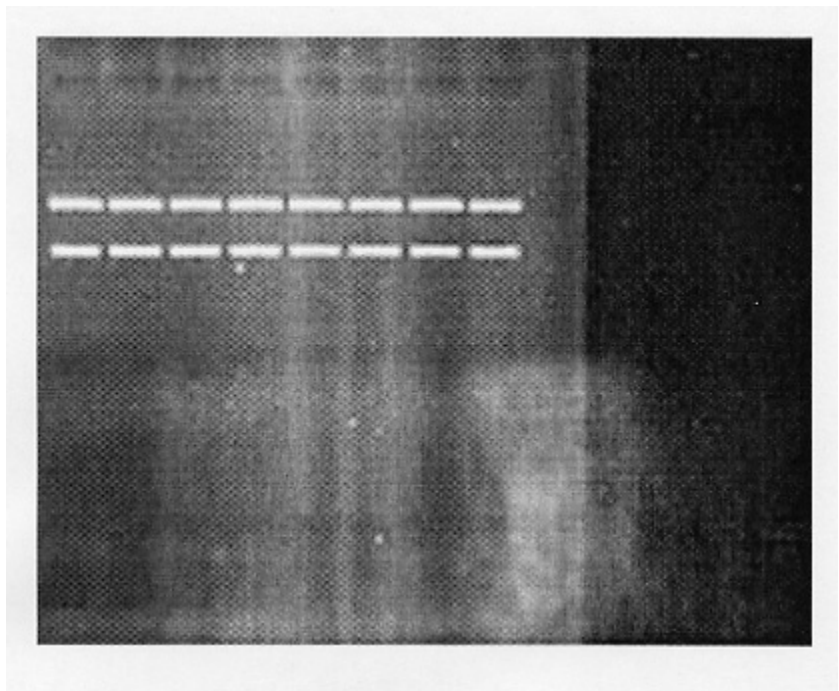
**Figure 4-1: CYP1A1 M1 MspI Restriction Digest**



**Figure 4-2: CYP1A1 M2 BsrDI Restriction Digest**



**Figure 4-3: CYP1A1 M3 MspI Restriction Digest**



**Figure 4-4: CYP1A1 M4 BsaI Restriction Digest**



**Table 4-2: Conditional logistic regression comparing cases and controls. Significant predictors of breast cancer risk [Numbers (N), Percentages (%)], [Means (S.D.)], age-adjusted odds ratio, 95% confidence interval**

Variable		Cases	Control	Odds ratio	95% CI
Family history of breast cancer	Yes	15 (6.00) <sup>a</sup>	1 (4.00) <sup>a</sup>	14.99	1.98,113.47
	No	235 (94.00) <sup>a</sup>	249 (96.00) <sup>a</sup>	1.00	
Waist/hip ratio (>0.90)	Yes	161 (64.40) <sup>a</sup>	117 (46.80) <sup>a</sup>	2.00	1.39,2.87
	No	89 (35.60) <sup>a</sup>	133 (53.20) <sup>a</sup>	1.00	
Abortion	Yes	18 (7.20) <sup>a</sup>	7 (2.80) <sup>a</sup>	2.43	1.01,5.85
	No	232 (92.80) <sup>a</sup>	243 (97.20) <sup>a</sup>	1.00	
Age at first full-term pregnancy		23.18 (5.36) <sup>b</sup>	21.87 (4.74) <sup>b</sup>	1.39	1.11,1.73
Education >= High School	Yes	136 (54.40) <sup>a</sup>	113 (45.20) <sup>a</sup>	1.31	1.07,1.61
	No	114 (54.60) <sup>a</sup>	137 (54.80) <sup>a</sup>	1.00	
Parity		4.1 (2.9) <sup>b</sup>	4.7 (3.1) <sup>b</sup>	0.88	0.81,0.96
Duration of breastfeeding		65.48 (47.32) <sup>b</sup>	80.96 (53.67) <sup>b</sup>	0.75	0.62,0.91

a: [Numbers (N), Percentages (%)], b: [Means (SD)]

**Table 4-3: CYP1A1 M1 Allele and Genotype frequencies for Breast Cancer Study Participants**

	Cases (n = 220)	Controls (n = 218)
All women		
Allele frequencies		
CYP1A1 M1 (T)	0.77	0.76
CYP1A1 M1 (C)	0.23	0.24
Genotype frequencies		
CYP1A1 M1 (T/T)	0.61	0.60
CYP1A1 M1 (T/C)	0.31	0.33
CYP1A1 M1 (C/C)	0.07	0.08
Premenopausal women		
Allele frequencies		
CYP1A1 M1 (T)	0.78	0.75
CYP1A1 M1 (C)	0.22	0.25
Genotype frequencies		
CYP1A1 M1 (T/T)	0.62	0.57
CYP1A1 M1 (T/C)	0.32	0.35
CYP1A1 M1 (C/C)	0.06	0.08
Postmenopausal women		
Allele frequencies		
CYP1A1 M1 (T)	0.75	0.77
CYP1A1 M1 (C)	0.25	0.23
Genotype frequencies		
CYP1A1 M1 (T/T)	0.60	0.62
CYP1A1 M1 (T/C)	0.30	0.31
CYP1A1 M1 (C/C)	0.10	0.07

**Table 4-4: Distribution of genotype frequencies of the CYP1A1 M1 polymorphism in relation to breast cancer risk**

	Cases (unmatched)	Controls (unmatched)	OR (95% CI)	OR (95% CI) <sup>a</sup>
All women				
CYP1A1 M1 (T/T)	135 (61.36)	130 (59.63)	1.00	1.00
CYP1A1 M1 (T/C)	69 (31.36)	71 (32.57)	0.78 (0.48-1.27)	0.79 (0.46-1.40)
CYP1A1 M1 (C/C)	16 (7.27)	17 (7.80)	0.79 (0.45-1.38)	0.91 (0.50-1.66)
CYP1A1 M1 (T/T)	135 (61.36)	130 (59.63)	1.00	
CYP1A1 M1 (T/C+C/C)	85 (38.63)	88 (40.37)	0.75 (0.48-1.18)	0.79 (0.48-1.29)
Pre-menopausal women				
CYP1A1 M1 (T/T)	78 (62.40)	71 (57.26)	1.00	
CYP1A1 M1 (T/C)	40 (32.00)	43 (34.68)	0.62 (0.31-1.24)	
CYP1A1 M1 (C/C)	7 (5.60)	10 (8.06)	0.63 (0.28-1.44)	
CYP1A1 M1 (T/T)	78 (62.40)	71 (57.26)	1.00	
CYP1A1 M1 (T/C+C/C)	47 (37.60)	53 (42.74)	0.58 (0.31-1.09)	
Postmenopausal women				
CYP1A1 M1 (T/T)	62 (60.19)	58 (61.70)	1.00	
CYP1A1 M1 (T/C)	31 (30.10)	29 (30.85)	1.00 (0.49-2.05)	
CYP1A1 M1 (C/C)	10 (9.71)	7 (7.45)	1.16 (0.55-2.44)	
CYP1A1 M1 (T/T)	62 (60.19)	58 (61.70)	1.00	
CYP1A1 M1 (T/C+C/C)	41 (39.81)	36 (38.30)	1.06 (0.55-2.01)	

a: adjusted for family history of breast cancer, waist/hip ratio, age at first childbirth and education

**Table 4-5: CYP1A1 M3 Allele and Genotype frequencies for Breast Cancer Study Participants**

	Cases (n = 229)	Controls (n = 227)
All women		
Allele frequencies		
CYP1A1 M3 (T)	0.88	0.87
CYP1A1 M3 (C)	0.12	0.13
Genotype frequencies		
CYP1A1 M3 (T/T)	0.77	0.76
CYP1A1 M3 (T/C)	0.23	0.23
CYP1A1 M3 (C/C)	0.004	0.01
Premenopausal women		
Allele frequencies		
CYP1A1 M3 (T)	0.87	0.85
CYP1A1 M3 (C)	0.13	0.15
Genotype frequencies		
CYP1A1 M3 (T/T)	0.75	0.71
CYP1A1 M3 (T/C)	0.24	0.28
CYP1A1 M3 (C/C)	0.01	0.01
Postmenopausal women		
Allele frequencies		
CYP1A1 M3 (T)	0.89	0.90
CYP1A1 M3 (C)	0.11	0.10
Genotype frequencies		
CYP1A1 M3 (T/T)	0.79	0.81
CYP1A1 M3 (T/C)	0.21	0.18
CYP1A1 M3 (C/C)	0.00	0.01

**Table 4-6: Distribution of genotype frequencies of the CYP1A1 M3 polymorphism in relation to breast cancer risk**

	Cases (unmatched)	Controls (unmatched)	OR (95% CI)	OR (95% CI) <sup>a</sup>
All women				
CYP1A1 M3 (T/T)	176 (76.86)	172 (75.77)	1.00	1.00
CYP1A1 M3 (T/C)	52 (22.71)	53 (23.35)	0.95 (0.62-1.47)	0.76 (0.47-1.22)
CYP1A1 M3 (C/C)	1 (0.44)	2 (0.88)	1.00 (0.25-4.00)	1.95 (0.24-6.01)
CYP1A1 M3 (T/T)	176 (76.86)	172 (75.77)	1.00	
CYP1A1 M3 (T/C+C/C)	53 (23.14)	55 (24.23)	0.95 (0.62-1.46)	0.76 (0.47-1.22)
Pre-menopausal women				
CYP1A1 M3 (T/T)	95 (75.40)	90 (71.43)	1.00	
CYP1A1 M3 (T/C)	30 (23.81)	35 (27.78)	0.81 (0.45-1.44)	
CYP1A1 M3 (C/C)	1 (0.79)	1 (0.79)	1.00 (0.25-4.00)	
CYP1A1 M3 (T/T)	95 (75.40)	90 (71.43)	1.00	
CYP1A1 M3 (T/C+C/C)	31 (24.60)	36 (28.57)	0.82 (0.46-1.43)	
Postmenopausal women				
CYP1A1 M3 (T/T)	81 (78.64)	80 (80.81)	1.00	
CYP1A1 M3 (T/C)	22 (21.36)	18 (18.18)	1.06 (0.54-2.10)	
CYP1A1 M3 (C/C)	0 (0.00)	1 (1.01)	-	
CYP1A1 M3 (T/T)	81 (78.64)	80 (80.81)	1.00	
CYP1A1 M3 (T/C+C/C)	22 (21.36)	19 (19.19)	1.06 (0.54-2.10)	

a: adjusted for family history of breast cancer, waist/hip ratio, age at first childbirth and education

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## **GENERAL DISCUSSION/FUTURE RESEARCH**

A rapidly growing global population, an aging world population profile with changing reproductive characteristics, a western-oriented diet heavily laden with animal fat, a sedentary lifestyle and a work environment exposed to various chemical carcinogens have all contributed partly to the rising global burden of breast cancer, a disease predominantly affecting the world's female sex. Breast cancer is the third most frequent cancer in the world (796,000 cases in 1990) and by far the most common malignancy of women (21% of all new cases). Worldwide, the ratio of mortality to incidence is about 61% (Parkin et al., 1999a). As a result, breast cancer ranks as the fifth cause of death from cancer overall, although it is still the leading cause of cancer mortality in women (the 314,000 annual deaths represent 14.1% of cancer deaths in females). Recent World Health Organization (WHO) statistics indicate that with an annual incidence rate increase of about 0.5% in western countries, and rates as high as 5% in China and other Eastern Asian countries and probably similar increases in sub-Saharan Africa and South America, the global burden of breast cancer in 2010 would be 1.45 million new cases, an 82% increase over the figure in 1990 (Parkin et al., 1999a). This increasing disease burden, likely to be born by the dwindling economies of the developing countries with the least resources to fight the disease calls for a reawakening of global efforts to combat the disease. We may not be able to completely eliminate breast cancer, but emerging evidence from the developed world provides evidence that we can at least slow the growth in the incidence of the disease and reduce morbidity and mortality in those afflicted by breast cancer.

Globally the prognosis for breast cancer is reported as generally rather good. The highest crude survival is reported by the SEER program, 84%, consistent with the age-adjusted estimate

for North America of 73%. Survival rates are high in Japan (74%) and Australia/New Zealand (68%) and lower in Europe (53% to 63%, consistent with the EURO CARE crude rate of 67%). Elsewhere survival ranges from 49% to 61% (Parkin et al., 1999a).

An issue of concern to the US in particular is the Black/White disparities in breast cancer burden within the US. Although, there are conflicting reports in the literature as to the degree of this disparity accounted for by racial differences in socioeconomic status, mammographic screening, and access to health care, there appear to be a consensus that a certain proportion remains explained by race. Black women diagnosed with breast cancer experience a 5-year relative survival of only 62%, compared with 79% for white women (Miller et al., 1992). Part of the poorer breast cancer survival for black women is due to more, advanced stage of disease at presentation, but some analyses that have controlled or stratified for stage have still found black women's survival to be poorer than that of their white counterparts (Rain et al., 1986; McWhorter et al., 1987; Kimmick et al., 1991; Fisher et al., 1993). Research concerning differences in breast cancer survival between black and white women suggests that the biological characteristics of tumors may differ between the races (Fisher et al., 1993; Dayal et al., 1982). Recent data from the National Cancer Institute Black/White Cancer Survival Study showed that after adjusting for stage, treatment, comorbid illness, and pathologic and sociodemographic variables, blacks continued to demonstrate a slightly increased, but not statistically significant, risk of death (hazard ratio=1.3; 95% CI 1.0-1.8) (Eley et al., 1994). Current efforts to eliminate health disparities among the different racial groups in the US will help show whether or not this Black/White differences will remain in the next decades to come.

A coalition of global efforts based upon sound public health principles and led by epidemiology hold the key to success in the fight to reduce the global burden of breast and other

cancers. The last half-century has seen the integration of disciplinary efforts to better understand the process of carcinogenesis. Recent advances in the traditional disciplines of bacteriology, virology, biochemistry and molecular biology and rapid progress in the Human Genome Project, and the application of tools of molecular biology to epidemiological studies will markedly influence the road map in the search for etiologic factors for breast cancer. For these efforts to have lasting impact on reducing global breast cancer burden, epidemiological research has to adopt a global approach. This is because there is evidence supporting a multifactorial etiology for breast cancer. In fact, it is being speculated that breast cancer is not a single disease entity but a final common pathway for the manifestation of a variety of genetic insults resulting in loss of cellular regulation and integration. While breast cancer occurs in all continents and countries across the globe, it is the nature of the disease that risk factors may differ from one part of the world to the other. Available data suggests that reproductive characteristics such as age at menarche, parity, duration of breastfeeding and practices such as use of exogenous estrogens for birth control and hormone replacement therapy (HRT) differ considerably between the developed world and developing countries (Collaborative Group on Hormonal Factors for Breast Cancer, 1997). In addition, prevalence of obesity (a risk factor associated with many chronic diseases including breast cancer) varies widely across the globe.

The choice of Nigerian women for this study was meant to serve dual purposes. Sub-Saharan Africa, of which Nigeria (population: 130 million) (Nigeria National Population Commission, 2003) is one of the component countries, is a region of the world grossly under-represented in the epidemiologic literature. Until recently, it used to be said that breast cancer was very rare in Nigeria; simply because the disease has not been adequately studied in the population. We believe that studying breast cancer risk factors among Nigerian women will not

only provide data about the disease among populations in sub-Saharan Africa but will contribute to global efforts at control and prevention. In addition, there is evidence that the Nigerian population under study share considerable genetic ancestry with Nigerian women. We suspect that predictors of breast cancer risk particularly genetic risks may be similar in the two populations. Studying breast cancer risk among African American women in the US has been hampered by low participation rate which has been attributed to factors such as mistrust and suspicion of the healthcare system, economic limitations, low level of education, concerns about drug toxicity, cultural/religious beliefs/attitudes, poor access to health care, lack of awareness about studies, past negative experiences, and physicians' attitude (Cox et al., 1998; Harris et al., 1996; Shavers-Hornaday et al., 1997; Swanson et al., 1995; Thomas et al., 1994; Robinson et al., 1996). Studying the role of low-penetrance genes such as cytochrome P450 (CYP1A1) and catechol-O-methyltransferase (COMT) gene polymorphisms in breast cancer susceptibility in Nigerian women provides an excellent opportunity for investigating the contribution of genetic factors to breast cancer burden in populations of African descent. The high participation rate of the study population is encouraging although infrastructural inadequacy creates setbacks in the conduct of such studies.

## **Article 1**

The first article evaluates the descriptive epidemiology of breast cancer risk in the study population. Breast cancer in our study population was found to be predominantly premenopausal (56.8%). Risk factors associated with increased risk of breast cancer in our study population include family history of breast cancer, higher waist/hip ratio, abortion, age at first full-term pregnancy, and higher level of education while increasing parity and longer duration of

breastfeeding conferred protection against the disease. These findings are similar to characteristics of the disease reported in studies comparing the risk profiles of breast cancer in African American and Caucasian women in the US. African American women under the age of 45 years have a greater incidence of breast cancer than Caucasian women in this young age range. These rates equalize during the fifth decade of life, and for women over the age of 55 years, incidence rates for Caucasian women surpass those of African Americans (Newman, 2005). Available clinical data indicate that 30%-40% of African American breast cancer patients seen in clinical practice are younger than 50 years, compared with 20% of Caucasian breast cancer patients in this age range (National Cancer Data Base, 1994). Factors accounting for this race-related risk profile are not clearly understood.

In a landmark study, Pike et al (1983) proposed the model of “breast tissue age” and this has been supported by a further extension of the Pike model by Pathaks and Whittemore (1992). The theory of Pike assumes that breast carcinoma vary in proportion to a power of the accumulated “breast tissue age” (Pike et al., 1983). In that model, the breast tissue ages at a constant rate between age at menarche and age at first full-term pregnancy (FFTP), at which time the hormonal milieu of pregnancy causes a one-time increase in breast tissue age but lowers the rate of subsequent “breast tissue aging”. This lower rate continues until the perimenopausal years, at which time it decreases linearly until menopause. After menopause the breast tissue continues to age, but at a much lower rate. This model predicts that at a given age, a woman with an FFTP in the preceding 5-10 years is at increased risk relative to a nulliparous woman. Studies of Janerich and Hoff (1982), and Pathak et al (1986) have confirmed this theory. Carrying a pregnancy to the third trimester confers a protective effect on lifetime risk for breast carcinoma if

that pregnancy occurs early in life. However, if a woman delivers her first child close to the age at which menopause occurs, her lifetime risk is actually higher than if she was nulliparous.

Pathaks and Whittemore (1992) extended Pike's single-birth model to a multiple birth model, by incorporating a smaller increase in risk at each additional full term pregnancy with a subsequent lowering of the rate at which breast tissue ages. Rosner et al (1994) fitted the extended Pike model to prospective data from the Nurse's Health Study and obtained breast carcinoma incidence curves for various combinations of age at FFTP, total parity, and ages at subsequent births. The predicted incidence curves for these hypothetical scenarios show lower risk for nulliparous women relative to multiparous women until ages 42-45 years, at which time a crossover occurs and the multiparous are at lower risk relative to the nulliparous women. The crossover for women with a single birth at age 20 years does not occur until age 55 years. This shift to a later age for the crossover effect for women with a single birth relative to multiparous women would be expected based on the hypothesized decreased rate of breast tissue aging after each subsequent pregnancy (Pathak et al., 2000).

Another factor that may contribute to higher breast cancer risk in young black women is the higher rate of abortions performed before the first full-term pregnancy and higher rate of use of oral contraceptive pills during the same period compared to young white women (Romieu et al., 1990). Although abortion was associated with increased risk of breast cancer in our patients, the abortion rates were low and data is lacking on the number of abortions performed before first full-term pregnancy in our patients. Increasing incidence of abortion and use of oral contraceptives among the younger cohorts of Nigerian women is of concern to the risk of breast cancer in the population in the future. Of concern also is the changing lifestyle of the younger cohorts of Nigerian women. Increasing westernization with more consumption of animal fat and sedentary lifestyle may lead to

obesity epidemic in the future as is being witnessed in the developed world. In addition, early menarche, delay in childbearing and reduced parity may add to drive up longer period of lifetime unopposed estrogen exposure (an established risk factor for breast cancer).

## **Article 2**

In the second article, we evaluated the association between the G to A transition polymorphism in codon 158 of the COMT gene and breast cancer risk in the study population. In the final conditional logistic regression model controlling for identified risk factors for breast cancer, harboring at least one low-activity COMT (Met) allele was associated with a significant 43% reduced risk of breast cancer (OR = 0.57, 95% CI 0.36-0.91). Similar reduced risks were observed in both premenopausal and postmenopausal breast cancer risk although further subgroup analysis were precluded by the low frequency of the COMT (Met) allele in the study population. We noted a 2 times lower frequency of the COMT (Met/Met) compared with the 13% reported in African Americans and 3-4 times lower than the 22-28% in Caucasians (Millikan et al, 1998). Our finding of reduced risk of breast cancer both in the final conditional logistic regression multivariate model and in univariate analysis in premenopausal and postmenopausal women agrees with the 20% reduced risk (although not significant) in both premenopausal and postmenopausal African American women and the 20% and 30% reduced risk (also not significant) of premenopausal and postmenopausal breast cancer risk among Caucasian women reported by Millikan et al (1998). The findings of 2-fold increased risk (not significant) of postmenopausal breast cancer among Caucasian women by Lavigne et al. (1997) and 5.7 fold increased risk of premenopausal breast cancer risk in Caucasian women by Thompson et al. (1998) is contrary to our findings. As noted previously, Asian women appear to



show a more consistent increased risk of breast cancer in carriers of the low-activity COMT (Met) allele (Yim et al., 2001).

Factors responsible for the inconsistencies in the reports in the literature with respect to association studies of the codon 158 COMT polymorphism are not easy to explain. For example, how do we explain the protection conferred by the COMT gene variant for breast cancer in our study subjects. In the first instance, this finding is contrary to our hypothesis of increased breast cancer risk among carriers of the variant allele because of the known functions of the COMT gene and the reported decreased methylation activity of the gene caused by the codon 158 polymorphism. We have already noted the much lower frequency of the COMT (Val/Met) and COMT (Met/Met) variants in our population compared with Caucasians. This reduced frequency of the variant allele might suggest a smaller impact of this polymorphism on the methylation of estrogen catechols in our subjects. Another possible explanation for our finding may be that the codon 158 Valine/Methionine polymorphism is in linkage disequilibrium with other polymorphisms of functional significance in the detoxification of biologically harmful estrogen catechols. We noted that sample size impacted subgroup analysis in our study. Although, our sample size of 231 cases and 229 controls with successful genotype data is larger than the sample size reported in most of the other studies noted above, the lower prevalence of the COMT (Met/Met) genotype in our population implies the need for larger sample size to be able to detect similar sizes of effects noted in either Caucasian or African American women. Using our COMT (Met/Met) frequency of 0.07 detected in our control subjects will require a sample size of 304 premenopausal cases and 304 premenopausal controls if we wish to detect an odd ratio of 2.4 (reported by Thompson et al., 1998). Our sample size of 142 premenopausal cases and 142 premenopausal controls is just about half the calculated sample size. Sample size is always an

issue of contention in early studies in population groups with no pre-existing data and our experience with the COMT gene highlights the importance of prior pilot studies. Our finding will serve this purpose for future disease association studies of this polymorphic variant in the Nigerian population.

Efforts to explain the discrepancy between the protective effect reported by Millikan et al. (1998) and the increased risk reported by Lavigne et al. (1997) and Thompson et al. (1998) (both reports contrasting with each other) among Caucasian women in the US have been less rewarding. As noted by Thompson et al. (1998), one possible explanation for the inconsistencies may be differences in the ethnic backgrounds of study participants. It should be noted that Caucasian Americans are of different European ethnicities which may affect the distribution of the COMT variant genotypes, a factor that can partly account for the divergent findings. Differences in these findings may also be the result of small sample size of the studies. As noted previously, the study of Lavigne et al. (1997) had only 113 cases and 113 controls in contrast to that of Thompson et al. (1998) (281 cases and 289 controls) and Millikan et al. (1998) (Caucasians: 389 cases and 379 controls, African Americans (265 cases and 263 controls). Another possible explanation may be the action of other compensating enzymes such as Glutathione S-transferase (GSTs), uridine diphosphoglucuronosyltransferase (UGT) 1A1, and the highly polymorphic manganese superoxide dismutase (MNSOD) genes that are induced when COMT activity is very low. Lower oxidative damage in the presence of COMT-(Met/Met) genotype may also be of importance; the level of 8-hydroxy-2'-deoxyguanosine in breast tissue was higher in patients with COMT (Val/Val) genotype as shown by Markides et al. (1998).

The fact that the COMT gene is highly polymorphic in some populations means that the population attributable risk for breast cancer resulting from the COMT genetic polymorphism may

be more marked in populations of higher prevalence of the homozygous COMT (Met/Met) genotype such as Caucasian populations (Millikan et al., 1998) compared with effects on risk in populations of lower prevalence as demonstrated in the Nigerian population and in Asian populations (Yim et al., 2001). In addition, environmental factors such as the prevalence of obesity in the population may also modulate the effect of the genotype on breast cancer risk. The implication of the above is that as part of the global strategy for the control and prevention there is need for studies of the prevalence of these polymorphisms in diverse population groups across the world to properly define the role of the COMT gene in breast cancer risk in various populations.

### **Article 3**

Our interest in the third article is to assess the role of four polymorphic variants (M1, M2, M3, and M4) in the cytochrome P450 (CYP1A1) gene in breast cancer susceptibility. CYP1A1 is involved in the 2-hydroxylation of estrogens to 2-hydroxy catechol metabolites for subsequent O-methylation to 2-methoxy derivatives including 2-methoxyestradiol. There is currently little information on the activities of these various polymorphic variants in the CYP1A1 gene in human populations, efforts are underway to characterize the biochemical properties of these variants to enhance research into their possible role in disease risk. There is evidence suggesting enhanced hydroxylation activity for the exon 7 polymorphism in some populations such as the Japanese (Nakachi et al., 1993) but not in African Americans (Taioli et al., 1995). This study has demonstrated the high prevalence of the CYP1A1 M1 and CYP1A1 M3 variants and the rarity of the CYP1A1 M2 and CYP1A1 M4 polymorphisms in the Nigerian population. The CYP1A1 M1 (C/C) genotype frequency of 8% was found to be twice the 3.5% reported in African Americans but about 3-4 times higher than the 2% noted in Caucasians (Taioli et al., 1995). Our finding of a

non-significant 19% reduced risk of breast cancer in our study subjects is in sharp contrast to the almost 10 times increased risk reported by Taioli et al. (1995) in African Americans although it must be pointed out that their small sample size of 21 cases and 85 cases creates room for spurious associations. Also, the finding of a significant 1.98 times increased risk of postmenopausal breast cancer risk in Chinese women in Taiwan (Huang et al., 1999) contradicts our finding of a non-significant 16% increased risk of breast cancer in postmenopausal women in our study. The finding of significant 40% and 42% reduced risk of breast cancer among Japanese women harboring the heterozygous and homozygous variants of this genotype respectively (Miyoshi et al., 2002) also reflects the inconsistencies associated with association studies between CYP1A1 M1 variant and breast cancer risk in various populations. It must however be appreciated that the 51% CYP1A1 M1 (T/C) heterozygosity and the 17% CYP1A1 M1 (C/C) homozygosity reported among Japanese patients is about twice the frequency of these genotypes in the Nigerian population.

Allele frequencies for the CYP1A1 M3 (T) and CYP1A1 M3 (C) alleles among our study participants was slightly lower than the figures reported by Crofts et al. (1993) among African American women. The CYP1A1 M3 variant is said to be African American-specific and has not been reported in Caucasians. We were unable to demonstrate any significant association between this novel polymorphism and breast cancer risk overall or in subgroup analysis in keeping with the findings of Taioli et al. (1995) who first described this variant in African American women although we noticed a higher frequency of the heterozygous CYP1A1 M3 (T/C) genotype compared with the figure reported by these investigators. We were unable to compute risks associated with the CYP1A1 M2 (Exon 7) and CYP1A1 M4 polymorphic variants due to their rarity in our population.

The interest in the 2-hydroxylation of estrogens has heightened with the recent finding that 2-methoxyestradiol, the O-methylation product of this pathway possesses anti-tumor properties. 2-Methoxyestradiol inhibits the proliferation of several cancer cell lines in vitro (Fotsis et al., 1994), and human breast cancer cell lines (estrogen receptor positive or negative) were particularly sensitive to a cytotoxic effect of 2-methoxyestradiol (Cushman et al., 1995). Additional studies indicated that 2-methoxyestradiol disrupted microtubule function and was a potent inhibitor of angiogenesis (Fotsis et al., 1994). By altering microtubule stability, inducing apoptosis and inhibiting angiogenesis in tumor cells and downregulating cytokine and PGE<sub>2</sub> induced in-situ aromatase synthesis in the breast (Purohit et al., 1999), 2-methoxyestradiol distinguishes itself as the new focus for new generation anti-cancer chemotherapy for both hormone dependent and hormone-independent breast cancer. In fact, 2-methoxyestradiol is undergoing a phase I trial as an angiogenesis inhibitor and search is ongoing for synthetic derivatives of this metabolite for anti-cancer chemotherapy (Brem et al., 1987).

Knowledge of estrogen metabolism has also provided new insight in appreciating the interaction between obesity and high fat diet in mediating breast carcinogenesis. It is now well documented that lean women or women on low fat diets have a lower risk of breast cancer than obese women or women consuming high fat diets. This finding is explained by the fact that in lean women or those on low fat diets, metabolism of estrogens via the 2-hydroxylation pathway predominates with subsequent formation of 2-methoxyestrogens (Longcope et al., 1987). Consequently, this causes downregulation of cytokine and PGE<sub>2</sub> receptors in breast adipose stromal cells and reduces in situ estrogen synthesis via peripheral aromatization. In contrast, in obese women or subjects on high fat diets that are associated with reduced synthesis of 2-methoxyestrogens, any cytokines or PGE<sub>2</sub> within the breast could result in increased production

of estrogens and subsequent risk of tumor development. There is also evidence suggesting that pesticides, which have been implicated in breast cancer, can also decrease estrogen 2-hydroxylation but increase 16-[alpha]-hydroxylation (Bradlow et al., 1995).

Some of the limitations of this study include recall and other biases associated with case control studies although it must be noted that genotype data is constant and unlikely to be affected by problems with recall. We calculated sample size for this study based on data on African American women; the genotype frequencies used for the calculation are similar to our findings among the Nigerian patients. Based on the genotype frequencies of the CYP1A1 M1 variant, we estimated that 35 CYP1A1 M1 (C/C) homozygous cases and controls would be required to demonstrate the odds ratio of 9.7 noted by Taioli et al (1995). Among our 220 cases and 218 control subjects with successful PCR-based RFLP assay, the number of cases and controls with the homozygous CYP1A1 M1 (C/C) genotype were 16 and 17 respectively, about half of the number calculated above. If the risk profile associated with the CYP1A1 M1 polymorphism reported by Taioli et al. (1995) were to be correct, we would have required twice our current sample size of 220 cases and 218 controls to detect similar effect size found in their study. However, the sample size of 21 cases and 85 controls recruited by Taioli et al. (1995) seem too small for conclusive statements to be made. In addition, theirs was probably the only study of the association of this polymorphism with breast cancer risk in populations of African descent.

To the best of our knowledge, ours is the first study to investigate the association between CYP1A1 genetic polymorphisms and breast cancer risk in indigenous African women. We have provided baseline data with which future studies can be compared. The distribution of the genotype frequencies of the CYP1A1 variants reported here will provide the basis for estimating

sample sizes required for further studies on the role of these variants on breast cancer risk in sub-Saharan African populations.

### **Future Research**

This study has provided us opportunity for an exploratory study to investigate the risk factors for breast cancer in Nigerian women, a population of exclusively indigenous African women, a population about whom little is known about breast cancer susceptibility. We have had a lot of difficulties; however we have learnt a lot of lessons within the past 3 years of this study. First, because of the multicenter design, we have been able to establish loci for future research on breast and other cancers within the Nigerian population. We have shown in the first article on the descriptive epidemiology of breast cancer in the population that reproductive variables and other factors similar to reports in other populations predict breast cancer risk in this population, thus establishing that lifetime estrogen exposure remains the main arbiter of breast cancer risk in indigenous African women as in other populations. Our exploratory data on the association between the COMT and CYP1A1 genetic polymorphisms and breast cancer risk in these women has provided a reference on which future studies would depend. We have pointed out limitations, which might have affected our results, and we hope to avoid them in future studies. The rapid progress of the Human Genome Project and accompanying fascinating progress in the health sciences including molecular epidemiology, molecular biology, genetic engineering, biostatistics and computer science and clinical medicine in the past half-decade has created an avalanche of opportunities for integrated efforts in the battle against breast cancer. Although it is becoming obvious that disease association studies based on single nucleotide polymorphisms are falling out of favor in molecular epidemiologic research, such studies have provided the impetus for the

future. We are moving to a stage where hard science combined with high through put research tools including microarray techniques and proteomics technology will drive the search for etiological factors in cancer and other chronic diseases. We hope to remain in the team of the future by building on the foundation provided by this exploratory study.

As a preliminary follow up to this study, we hope to further explore the association between CYP1A1 genetic polymorphisms and breast cancer risk using the haplotype approach combining allele and genotype data for CYP1A1 M1, CYP1A1 M2, CYP1A1 M3, and CYP1A1 M4 derived from this study. We also hope to apply the same technique in evaluating the association between CYP1B1 M1, CYP1B1 M2, CYP1B1 M3, and CYP1B1 M4 polymorphic variants of the cytochrome P4501B1 (CYP1B1) gene and breast cancer risk in this population. We have generated allele frequency and genotype data for the tetranucleotide simple tandem repeat polymorphism in the 3' noncoding region of the aromatase gene. We will also evaluate the association between repeats lengths and breast cancer risk in our study population. By combining the genotype and haplotype data, we hope to build a multigenic model of breast cancer risk in these subjects. Polymorphisms in other relevant genes in the pathway of estrogen synthesis and metabolic pathway including the CYP17 beta-hydroxysteroid dehydrogenase (CYP17), cytochrome P450 3A4 (CYP3A4), glutathione-S-transferase (GSTs), uridine diphosphoglucuronosyltransferase (UTG) 1A1, N-acetyltransferases (NATs) and manganese superoxide dismutase (MNSOD) genes will be investigated as part of the ongoing study. We will also examine polymorphisms in the estrogen receptor polymorphisms and variations in DNA repair genes including XRCC1, and the effect of epigenetic modifications such as promoter hypermethylation of various genes in transcriptional silencing and how this relates to breast cancer risk. As mentioned previously, there is need to expand the sample size of our study to



increase its robustness for multigenic modeling. We hope to achieve this by recruiting more study participants from the Nigerian institutions; a sample size of 1000 cases and 1000 control subjects is being estimated for this future study. We hope to expand our capacity for robust comparisons by collaboration with other investigators with similar interests working with US populations as well as populations of African descent in the Caribbean.

## **PUBLIC HEALTH SIGNIFICANCE OF STUDY**

This study was focused on identifying risk factors for breast cancer in Nigerian women, an indigenous sub-Saharan African population. The results have demonstrated that sociodemographic characteristics, reproductive variables and anthropometric measures are significant predictors of breast cancer risk in the study population. In particular, family history of breast cancer in first- and second-degree relatives, higher level of education, waist/hip ratio, abortion and later age at first full-term pregnancy were associated with increased risk of breast cancer while high parity and long duration of breastfeeding conferred protection against the disease in Nigerian women. We further evaluated the contribution of polymorphisms in catechol-O-methyltransferase (COMT) and cytochrome P450 1A1 (CYP1A1) genes in breast cancer susceptibility among the study population. Our results provide evidence suggesting that harboring at least one low activity COMT (Met) allele of the codon 158 Valine to Methionine polymorphism of the COMT gene significantly reduced the risk of breast cancer. The study also demonstrated the rarity of the CYP1A1 M2 and CYP1A1 M4 polymorphisms of the CYP1A1 gene in the study population. Although the CYP1A1 M1 and CYP1A1 M3 genes were found to be highly polymorphic, we were unable to demonstrate significant association between these polymorphisms and breast cancer risk in the study population.

While these variables are similar to the risk factors for breast cancer described in African American women and US Caucasians and other populations across the globe, we recognized notable exceptions. Firstly, there was a preponderance of premenopausal breast cancer among the study population similar to the distribution of the disease in African American women but in contrast to the higher proportion of postmenopausal breast cancer in Caucasian populations.

Secondly, the use of oral contraceptives and postmenopausal hormone replacement therapy was very low among the Nigerian women in this study in contrast to the higher figures reported in Western populations. Thirdly, the Nigerian women in this population were predominantly lean population with mean body mass index (BMI) below figures reported in the developed countries. Fourthly, the frequency of the COMT (Met) allele of the codon 158 polymorphism (Valine to Methionine substitution) of the COMT gene was lower in our study population compared to figures reported in African American women and Caucasians in the US. Also the proportion of the homozygous genotype of the African American-specific CYP1A1 M3 polymorphism of the CYP1A1 gene was found to be higher in the Nigerian population than the reported figures in African American women.

Although, it is consolatory to note that the epidemic of global obesity has not spread to sub-Saharan Africa, there is need for policy guidelines necessary to prevent the obesity phenomenon from spreading to the developing countries. In addition, the adoption of abortion as means of family planning should be discouraged; effort should be directed to alternative methods that do not endanger the life of women through increasing breast cancer risk. Also, physical inactivity appear to be creeping into the lives of populations of developing countries as sedentary office jobs replace the traditional agrarian economies. Alcohol consumption and cigarette smoking rates although low in the study population are likely to be on the increase in these populations in this century. Both factors have been linked to increased risk of breast cancer in developed Western populations. The global anti-smoking campaign should be expanded to the developing countries as the Tobacco companies shift their operations to these countries.

Overall, the findings of this study has contributed to knowledge about risk factors for breast cancer in populations of African ancestry in general and Nigerian population in particular and has provided baseline data for future epidemiologic studies in sub-Saharan African populations.

## **SUMMARY**

The study titled “Estrogens, Genetic Polymorphisms and Breast Cancer Risk in Nigerian Women” was designed to evaluate the role of reproductive variables (surrogate measures of lifetime estrogen exposure), family history, anthropometric variables and genetic polymorphisms in breast cancer susceptibility in Nigerian women. The first article of the study evaluated the descriptive epidemiology of breast cancer. Two hundred and fifty women with histologically-confirmed breast cancer and their age matched controls were recruited from four University Teaching Hospitals in Midwestern and Southeastern Nigeria for the study. Recruitment was successfully conducted during a 20-month period between September 2002 and April 2004. The association between various reproductive variables, sociodemographic characteristics and anthropometric measurements and breast cancer risk in the study population was assessed using conditional logistic regression models.

Results showed that a positive family history of breast cancer was associated with increased risk of breast cancer. Higher level of education above High School also increased the risk of breast cancer in these women. While reproductive variables such as late age at first full-term pregnancy and abortion were associated with increased risk of breast cancer, high parity and long duration of breastfeeding were protective against the development of the disease. Among the anthropometric measurements evaluated for association with breast cancer risk, only waist/hip ratio was found to be significantly associated with increased breast cancer risk. The population was found to be lean with mean body mass index (BMI) below figures reported in

most Western populations. Although there was a tendency towards increased risk with increasing height, the difference did not attain statistical significance.

The second article was designed to investigate the hypothesis that harboring the low-activity COMT (Met) allele of the codon 158 valine to methionine substitution polymorphism of the catechol-O-methyltransferase (COMT) gene will increase the risk of breast cancer. Although biological samples were obtained from the 250 women with breast cancer and the 250 age-matched controls recruited from the Nigerian institutions, the polymerase chain reaction (PCR)-based restriction length polymorphism (RFLP) assay was successful in 231 cases and 229 control subjects. It was found that the frequency of the COMT (Met) allele was lower in the Nigerian study population with a correspondingly higher prevalence of the COMT (Val) allele compared to the reported distribution of these alleles in both African American and Caucasian women in the US. After controlling for risk factors for breast cancer identified in the descriptive analysis, it was found that harboring at least one low activity COMT (Met) allele of the COMT polymorphism (COMT valine to methionine substitution in codon 158) was associated with significant decrease in breast cancer risk in the final multivariate logistic regression model. Although, this is contrary to our initial hypothesis, we speculate that this polymorphism may be in linkage disequilibrium with other yet to be identified polymorphisms that might be associated with reduced breast cancer risk in our study population.

The third article investigated the association between four polymorphisms in the cytochrome P450 1A1 (CYP1A1) gene and the risk of breast cancer in the study population using a PCR-based RFLP assay. The assays were successful in 220 cases and 218 controls. While the CYP1A1 M1 and CYP1A1 M3 polymorphisms were found to be common in our study populations, the CYP1A1 M2 and CYP1A1 M4 variants were found to be very rare as only one

control subject carried the heterozygous CYP1A1 M2 (A/G) genotype. All the study participants were homozygous for the CYP1A1 M4 (C) allele.

A conditional logistic regression model was used to evaluate the association between these polymorphisms and breast cancer risk. We were unable to demonstrate significant association between the CYP1A1 M1 and CYP1A1 M3 polymorphisms and breast cancer risk, both overall and in subgroup analysis based on menopausal status. We however noted a higher frequency of the African American-specific CYP1A1 M3 (C) allele in our study subjects compared with reported figures in African American women.

Results of this study have important public health implications. First, it has provided evidence for a role for reproductive and sociodemographic variables in susceptibility to breast cancer in indigenous African women; thus contributing to the global epidemiologic literature on risk factors for breast cancer in populations of African ancestry. It has also provided data suggesting protection for breast cancer for women harboring at least one low activity COMT (Met) allele of the COMT gene. As noted previously, the study provides baseline data that will serve as an important resource tool for similar epidemiologic studies in sub-Saharan African populations. In particular, it provides genotype data for the determination of sample size in studies evaluating the role of polymorphisms in the COMT and CYP1A1 genes and breast cancer risk. In addition, the findings of this study will serve a useful resource tool in policy decisions aimed at breast cancer control and prevention in these populations.

## APPENDIX A: STUDY QUESTIONNAIRE

Date \_\_\_\_/\_\_\_\_/\_\_\_\_\_  
Day Mon Year

ID No. NB02 \_\_\_\_\_  
IRB # 010767  
Approved \_08\_/28/\_01\_  
Renewal 08/27/02

Data Status:

1. Final complete \_\_\_\_\_ 2. Final incomplete \_\_\_\_\_

### ESTROGENS, GENETIC POLYMOPHISMS AND BREAST CANCER RISK Baseline Questionnaire

Consent form signed: 1. No \_\_\_\_ 2. Yes \_\_\_\_

IF NO, DO NOT PROCEED UNTIL STUDY HAS BEEN EXPLAINED AND CONSENT FORM HAS BEEN SIGNED.

Please complete:

(Note: You need not answer questions you feel uncomfortable answering)

Date of Birth: \_\_\_\_/\_\_\_\_/\_\_\_\_\_  
Day Month Year

Do you wish to participate in this study?

1. No \_\_\_\_ 2. Yes \_\_\_\_

If yes, previously diagnosed with breast cancer 1. No \_\_\_\_ 2. Yes \_\_\_\_

Status: 1. Agrees to participate 2. Undecided/postponed 3. Withdrawn \_\_\_\_\_

---

### FOR OFFICE USE ONLY

Interviewer ID# \_\_\_\_\_ Nigerian referral source \_\_\_\_\_

Data Entry Date (1) \_\_\_\_/\_\_\_\_/\_\_\_\_ Data Entry ID# \_\_\_\_\_  
(2) \_\_\_\_/\_\_\_\_/\_\_\_\_

---



THE FOLLOWING QUESTIONS ASK ABOUT YOUR GENERAL BACKGROUND, WORK HISTORY, AND SMOKING HISTORY.

1. Place of Birth - Country of Birth? \_\_\_\_\_

2a. What tribe/ethnic group are you?

Edo Bini/Benin\_\_\_\_ E.Ishan\_\_\_\_ E.Ora/Owan\_\_\_\_ E.Etsako/Afemai\_\_\_\_ E.Akoko\_\_\_\_ Urhobo\_\_\_\_  
Itsekiri\_\_\_\_ Ijaw/Izon\_\_\_\_ Ibo\_\_\_\_ Bendel\_\_\_\_ I.Imo/Anambra/Ebonyi/Abia\_\_\_\_ Efik\_\_\_\_ Kalabari\_\_\_\_  
Yoruba\_\_\_\_ Other(Specify)\_\_\_\_\_

2b. What is your religion? \_\_\_\_\_

3. What is the highest grade or level of schooling you completed? (Mark only one response)\_\_\_\_\_

- |  |                        |
|--|------------------------|
| 1. Less the 8 years  | 5. Some university     |
| 2. 8 thru 11 years   | 6. University graduate |
| 3. 12 years or completed secondary school  | 7. Postgraduate        |
| 4. Post secondary training other than university<br>(vocational or technical training) |                        |

4. What is your current marital status? (Check only one; if common-law marriage, check 1.)\_\_\_\_\_

- |                                 |                  |
|---------------------------------|------------------|
| 1. Married or living as married | 4. Separated     |
| 2. Widowed                      | 5. Never married |
| 3. Divorced                     |                  |

5. Which of these categories best describes your current working situation? \_\_\_\_\_

- |               |                          |
|---------------|--------------------------|
| 1. Homemaker  | 5. Extended sick leave   |
| 2. Working    | 6. Disabled              |
| 3. Unemployed | 7. Other (specify) _____ |
| 4. Retired    |                          |

6. What has been your usual adult occupation? That is, at what type of occupation have you worked the longest during your adult life?

Usual adult occupation: \_\_\_\_\_

7. What were your usual activities and duties in this occupation?

Usual activities or duties: \_\_\_\_\_

8. In what type of business or industry were you usually employed in this occupation?

Business or industry: \_\_\_\_\_

9. How many years have you worked in this occupation?

\_\_\_\_\_ Number of years worked in occupation

10. List other jobs you have had: \_\_\_\_\_

11. Have you ever worked as an agricultural worker or pesticide worker? \_\_\_\_\_

1. No (Go to Question 11j)

2. Yes

11b. If yes, circle your years of age when you worked as an agricultural worker/grounds keeping more than one month? (For example, if the man worked on a farm from ages 18-25, and age 45-51, draw a circle around the numbers 18 19 and a circle around 20 21 22 23 24 25 and a circle around 45 46 47 48 49 and a circle around 50 51.)

List crops or livestock tended.

10 11 12 13 14 15 16 17 18 19

\_\_\_\_\_

20 21 22 23 24 25 26 27 28 29

\_\_\_\_\_

30 31 32 33 34 35 36 37 38 39

\_\_\_\_\_

40 41 42 43 44 45 46 47 48 49

\_\_\_\_\_

50 51 52 53 54 55 56 57 58 59

\_\_\_\_\_

60 61 62 63 64 65 66 67 68 69

\_\_\_\_\_

70 71 72 73 74 75 76 77 78 79

\_\_\_\_\_

80 81 82 83 84 85 86 87 88 89

\_\_\_\_\_

11c. Were chemical fertilizers used on the estate/farm/holding/plantation where you worked? \_\_\_\_\_

1. No (Go to Question 11f)

2. Yes

3. Don't know (go to question 11f)

11d. If yes, circle all age periods when you worked on an estate/farm/holding/plantation when chemical fertilizers were used.

Age periods

10-19

30-39

50-59

70-79

20-29

40-49

60-69

80 and older

11e. What chemical fertilizers were used?

---



---



---

11f. Were pesticides used on the estate/farm/holding/plantation where you worked? \_\_\_\_

1. No (Go to Question 11j)                      2. Yes                      3. Don't know (Go to Question 11j)

11g. Circle all age periods when you worked on an estate/farm/holding/plantation when pesticides were used.

Age periods                      10-19                      30-39                      50-59                      70-79

20-29                      40-49                      60-69                      80 and older

11h. What chemical pesticides were used when you worked on an estate/farm/holding/plantation?

---



---



---

11i. Circle your years of age when you worked as a pesticide sprayer in agriculture, grounds keeping, business or residential work for more than one month? (For example, if the man worked with pesticides from ages 18-25, and age 45-51, draw a circle around the numbers 18 19 and a circle around 20 21 22 23 24 25 and a circle around 45 46 47 48 49 and a circle around 50 51.)

List pesticides used.

10	11	12	13	14	15	16	17	18	19	_____
20	21	22	23	24	25	26	27	28	29	_____
30	31	32	33	34	35	36	37	38	39	_____
40	41	42	43	44	45	46	47	48	49	_____
50	51	52	53	54	55	56	57	58	59	_____
60	61	62	63	64	65	66	67	68	69	_____
70	71	72	73	74	75	76	77	78	79	_____
80	81	82	83	84	85	86	87	88	89	_____

11j. Check the animals you keep?

- |                    |               |
|--------------------|---------------|
| 1. ____ chickens   | 5. ____ cows  |
| 2. ____ ducks      | 6. ____ goats |
| 3. ____ pigeons    | 7. ____ other |
| 4. ____ other fowl |               |

12. Have you ever smoked cigarettes regularly for six months or longer? \_\_\_\_

1. No (Go to Question 20)                      2. Yes

13. At what age did you start smoking cigarettes regularly? \_\_\_\_  
(Enter age first started smoking in the space provided)

14. Do you smoke cigarettes regularly now? \_\_\_\_

1. No    2. Yes (Go to Question 16)

15. At what age did you last stop smoking cigarettes regularly? \_\_\_\_  
(Enter age last stopped smoking in the space provided)

16. During periods when you smoked, how many cigarettes did or do you usually smoke per day? \_\_\_\_

- |          |          |          |               |
|----------|----------|----------|---------------|
| 1. 1-10  | 3. 21-30 | 5. 41-60 | 7. 81 or more |
| 2. 11-20 | 4. 31-40 | 6. 61-80 |               |

17. During periods when you smoked, did or do you more often smoke filter or non-filter cigarettes? \_\_\_\_

1. Filter more often                      2. Non-filter more often                      3. Both about equally

18. Do you now or did you ever smoke a pipe regularly for a year or longer? \_\_\_\_

- |  |                              |
|--|------------------------------|
| 1. Never smoked a pipe                         | 3. Currently do smoke a pipe |
| 2. Did smoke a pipe but currently do not smoke |                              |

19. Do you now or did you ever smoke cigars regularly for a year or longer?

- |  |                              |
|--|------------------------------|
| 1. Never smoked cigars                         | 3. Currently do smoke cigars |
| 2. Did smoke cigars but currently do not smoke |                              |

ID No. NB02 \_\_\_\_\_

20a. Do you drink alcohol? Yes\_\_\_\_(1) No\_\_\_\_(2)

b. which type of alcohol do you usually take?

1. Beer\_\_\_\_\_ 2. Palm wine\_\_\_\_\_ 3. Ogogoro/whiskey/Liquor\_\_\_\_\_ 4. wine\_\_\_\_\_

c. How often do you drink alcohol?

1. Daily\_\_\_\_\_ 2. Two to 3 times a week\_\_\_\_\_ 3. Less than twice a week 4. Occasionally\_\_\_\_\_

21. What is your position among your mother's children (both alive and late)?

22a. Number of whole sisters		Number of half sisters		22b. Number of whole brothers		Number of half brothers	
# Living	# Deceased	# Living	# Deceased	# Living	# Deceased	# Living	# Deceased

22c. Number of whole sisters who live in:		22d. Number of half sisters who live in:	
Benin-city or w/I 10 km	Elsewhere in Nigeria	Benin-City or w/I 10km	Elsewhere in Nigeria

THE FOLLOWING QUESTIONS ASK ABOUT YOUR FAMILY MEDICAL HISTORY AND YOUR PERSONAL MEDICAL HISTORY.

22c. Is your mother living?\_\_\_\_\_

1. No, deceased

2. Yes living in/near Benin-City.

3. Yes living elsewhere in Nigeria

4. Yes, living outside Nigeria

23a. Have parents, children, brothers, sisters, half-brother, or half-sisters ever been diagnosed as having any type of cancer? (Do not include Basal-Cell Skin Cancer)

1. No (Go to question 23c)                      2. Yes

23b. Please complete this chart for each relative (mother, father, children, brothers, sisters, half-brother, or half-sisters) diagnosed with cancer? (Do not include Basal-Cell Skin Cancer) (If you have more than four relatives diagnosed with cancer, please include a separate page with this information.)

Male/Female	Type of cancer	Age at diagnosis
1 <sup>st</sup> Relative	_____	_____
2 <sup>nd</sup> Relative	_____	_____
3 <sup>rd</sup> Relative	_____	_____
4 <sup>th</sup> Relative	_____	_____

23c. Have any of your relatives been diagnosed with fibrocystic disease of the breast? Yes\_\_\_  
No\_\_\_

24. What is or was your weight at these ages? (Enter the weight in pounds in the space provided)

Weight at Age 50? \_\_\_ \_\_\_ \_\_\_ Weight at Age 20?\_\_\_ \_\_\_ \_\_\_ Current Weight?\_\_\_ \_\_\_ \_\_\_

25. How tall are you? (Record your height in feet and inches in the space provided.)

Feet \_\_\_\_\_ Inches \_\_\_\_\_

26. During the last 12 months, have you regularly used aspirin or aspirin-containing products, such as Bufferin, Anacin, cafenol, disprin, phensic, alka seltzer? (Please do not include aspirin-free products such as Tylenol and Panadol.) \_\_\_\_\_ 1. No (Go to Question 28)                      2. Yes

27. During the last 12 months, how many pills of aspirin or aspirin-containing products did you usually take per day, per week, or per month? \_\_\_\_\_

- |                      |                          |                  |
|----------------------|--------------------------|------------------|
| 1. 1 per day         | 4. 2 per week            | 7. 2-3 per month |
| 2. 2 or more per day | 5. 3-4 per week          |                  |
| 3. 1 per week        | 6. Less than 2 per month |                  |

28. During the last 12 months, have you regularly used ibuprofen-containing products, such as Advil, Nuprin, Motrin, olfen, feldene, indocin? \_\_\_\_\_

1. No (Go to Question 30)                      2. Yes

29. During the last 12 months, how many pills of ibuprofen-containing products did you usually take per day, per week, or per month? \_\_\_\_\_

- |                      |                          |                  |
|----------------------|--------------------------|------------------|
| 1. 1 per day         | 4. 2 per week            | 7. 2-3 per month |
| 2. 2 or more per day | 5. 3-4 per week          |                  |
| 3. 1 per week        | 6. Less than 2 per month |                  |

30. Has the doctor ever told you that you have any of the following conditions?  
(Mark Yes, No or Don't Know for each condition)

1	2	3
No	Yes	Don't Know
---	---	----- High blood pressure (hypertension)
---	---	----- Coronary heart disease/heart attack
---	---	----- Stroke
---	---	----- Emphysema
---	---	----- Chronic bronchitis
---	---	----- Diabetes
---	---	----- Colorectal polyp(s)
---	---	----- Arthritis
---	---	----- Osteoporosis
---	---	----- Hepatitis
---	---	----- Cirrhosis
---	---	----- Diverticulitis/diverticulosis
---	---	----- Gall bladder stones or inflammation

31. Have you ever been diagnosed as having breast cancer?

1. No (Go to Question 33)                      2. Yes, age at diagnosis \_\_\_\_\_

32. Have you ever been diagnosed with any type of cancer other than breast cancer? (Do not include Basal-Cell Skin Cancer).

What type of cancer did you have?		How old were you when you were diagnosed with this cancer?
1st Cancer	_____ Type of cancer	_____ Age
2nd Cancer	_____ Type of cancer	_____ Age
3rd Cancer	_____ Type of cancer	_____ Age

33. At what age did you first began your menstrual period? \_\_\_\_\_

34a. Are your menstrual periods regular?    1. No\_\_\_\_\_    2. Yes\_\_\_\_\_

34b. Date of Last Menstrual Period    \_\_\_\_\_

35a. Have you ever (or are you currently) taking female hormones for birth control?

1. No\_\_\_\_\_    2. Yes\_\_\_\_\_    3. Don't know\_\_\_\_\_



## b. Dates or time period, name of pill

Name of birth control pill	Age at onset of use	Duration of use (months)

36a. Have you ever taken female hormones other than for birth control? 1. No\_\_\_\_\_ 2. Yes\_\_\_\_\_  
3. Don't know\_\_\_\_

b. Dates or time period, name and  
reason:\_\_\_\_\_

37.a. At what age did you experience menopause (absence of menstrual period for more than 1 year)?

\_\_\_\_\_ Years

b. Did you receive any medications because of menopause?

1. No\_\_\_\_\_ 2. Yes\_\_\_\_\_ 3. Don't know\_\_\_\_\_

If the subject indicates that medication was received because of menopause, complete questions 37c and 37d. If the subject did not receive any medication because of menopause, continue with question 38a.

c. What type of medicine?

(List names of medications below when possible)

Tranquilizer? 1. No\_\_\_\_\_ 2. Yes\_\_\_\_\_ 3. Don't  
know\_\_\_\_\_

Pain Killer 1. No\_\_\_\_\_ 2. Yes\_\_\_\_\_ 3. Don't  
know\_\_\_\_\_

Female Hormones 1. No\_\_\_\_\_ 2. Yes\_\_\_\_\_ 3. Don't know\_\_\_\_\_

d. How many months have you taken female hormones

Name of Hormone (HRT)	Age at onset of use	Duration of use (months)

e. Are you currently taking the female hormones? 1. No\_\_\_\_\_ 2. Yes\_\_\_\_\_

38a. Have you had any of the following operations?

Caesarean section (operation for delivery) 1. No\_\_\_\_\_ 2. Yes\_\_\_\_\_ 3. Don't know\_\_\_\_\_

Tubal ligation (tubes tied for family planning) 1. No\_\_\_\_\_ 2. Yes\_\_\_\_\_ 3. Don't know\_\_\_\_\_

Hysterectomy (womb removed at operation) 1. No\_\_\_\_\_ 2. Yes\_\_\_\_\_ 3. Don't know\_\_\_\_\_

If the subject indicates that she had hysterectomy, complete questions 38b. If the subject did not have hysterectomy, continue with question 38c.

b. Did they remove the ovaries with the womb? 1. No\_\_\_\_\_ 2. Yes\_\_\_\_\_ 3. Don't know\_\_\_\_\_

c. What type of medicine were you given after the operation? (List the names of the medications when possible).

Pain Killer? 1. No\_\_\_\_\_ 2. Yes\_\_\_\_\_ 3. Don't know\_\_\_\_\_

Antibiotics? 1. No\_\_\_\_\_ 2. Yes\_\_\_\_\_ 3. Don't know\_\_\_\_\_

Female Hormones? 1. No\_\_\_\_\_ 2. Yes\_\_\_\_\_ 3. Don't know\_\_\_\_\_

d. How many months have you taken female hormones? \_\_\_\_\_months

e. Are you currently taking female hormones? 1. No\_\_\_\_\_ 2. Yes\_\_\_\_\_

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39a. How many children do you have? \_\_\_\_\_

39b. History of childbirths/pregnancies and breastfeeding (Include all abortions, indicate whether spontaneous or induced).

Child/pregnancy No.	Date of birth	Duration of pregnancy	Duration of breastfeeding (months)

40. At what age did you have your first live birth \_\_\_\_\_

41. At what age did you have your first pregnancy over 6 months (alive or late)? \_\_\_\_\_

42a. Did you breastfeed? 1. No \_\_\_\_\_ 2. Yes \_\_\_\_\_

b. How many months did you breastfeed your children? \_\_\_\_\_

43. Have you had any of the following surgical procedures of the breast?

- |                                 |                                   |
|---------------------------------|-----------------------------------|
| 1. Biopsy                       | 4. Breast surgery, type unknown   |
| 2. Surgery for benign disease   | 5. None (Go to Question 45)       |
| 3. Mastectomy for breast cancer | 6. Don't know (Go to Question 45) |

44. How old were you when you had a surgical procedure for breast cancer for the first time?

- |                 |          |             |
|-----------------|----------|-------------|
| 1. Less than 30 | 3. 40–49 | 5. 60–69    |
| 2. 30–39        | 4. 50–59 | 6. 70–older |

45a. During the past year, have you had a breast examination?

1. No \_\_\_\_\_ 2. Yes, once \_\_\_\_\_ 3. Yes, more than once \_\_\_\_\_ 4. Don't know \_\_\_\_\_

b. During the past month, have you done a Self–breast examination?

1. No \_\_\_\_\_ 2. Yes, once \_\_\_\_\_ 3. Yes, more than once \_\_\_\_\_ 4. Don't know \_\_\_\_\_

46. How would you rate your awareness of breast disease? (e.g. painful/painless breast lump, bloody nipple discharge or ulceration)?

1. Very high \_\_\_\_\_ 2. High \_\_\_\_\_ 3. Low \_\_\_\_\_ 4. Very low

47. What is the date you completed this questionnaire? \_\_\_\_\_/\_\_\_\_\_/\_\_\_\_\_

Day Month Year

Following Measurements with light clothing, without shoes

48. Height \_\_\_\_\_cm      \_\_\_\_\_' \_\_\_\_\_"

49. Weight \_\_\_\_\_kg      \_\_\_\_\_' \_\_\_\_\_"

50. Waist \_\_\_\_\_cm (at umbilicus) \_\_\_\_\_' \_\_\_\_\_"

51. Hips \_\_\_\_\_cm (at widest part of buttocks) \_\_\_\_\_' \_\_\_\_\_"

## APPENDIX B: CONSENT FORM



University of Pittsburgh  
Graduate School of Public Health

IRB # 010767

NB0 2 \_ \_ \_ \_ \_

### CONSENT TO ACT AS A SUBJECT IN A CLINICAL STUDY

Title: Estrogens, Genetic Polymorphisms and Breast Cancer Risk

INVESTIGATORS: University of Pittsburgh, Pittsburgh, Pennsylvania 15261, USA

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Surgeon.

SOURCE OF SUPPORT: U.S. Department of Defense (Pending)

DESCRIPTION: You are being asked to participate in a genetic research study involving breast cancer. We want to study the genetic factors in the body, called genes or DNA, which may help cause breast cancer in some women. We plan to compare these genes in women who have breast Cancer with these genes in women who do not have breast cancer. You are being asked to be in the study because you have breast cancer or because you are a hospital patient without breast cancer. The study will require one visit. At the visit, a breast examination will be conducted. If you do not have cancer and the doctor finds that your breast has a lump, you will not be eligible

to continue in the study. If you are eligible to continue, we will record your height, weight, and other physical measurements. We will also ask questions from the Breast Cancer Questionnaire about your history of cancer, and medical, work, and smoking history. This will take about 30 minutes.

Blood and urine samples will be collected from you to test for factors, e.g. female hormones, related to breast cancer. Female hormones are substances in the body which are related to the female organs, e.g. the breasts, ovaries and uterus. The DNA tests will be done on the blood sample. A vial with a needle at the top will be stuck into a vein in your arm to draw a small blood sample (about four teaspoons). Part of your blood and urine samples will be stored indefinitely at the University of Pittsburgh for possible future research tests to help understand the causes of breast cancer.

If you have breast cancer, we also request your permission for us to review your medical record and obtain a copy of the report of the doctor's examination of your breast tissue under the microscope at the pathology department of University of Benin Teaching Hospital/Nnamdi Azikiwe University Teaching Hospital/University of Nigeria Teaching Hospital/University of Port Harcourt Teaching Hospital. This review of your medical record will be completed within the next year.

If you agree to participate in the research project, use of your biological and genetic material will be under the control of the principal investigator of this research project.

**RISKS AND BENEFITS:** There are no benefits for participation in this study. Your blood will be taken from your arm by one of the local doctors working on the study. There may be a little discomfort or a bruise on your arm. You may feel dizzy or faint, but this would be rare. The breast examination will be performed by a specialist surgeon.

The DNA tests and other future research tests are not ordinary medical tests which doctors use to help decide your care. Instead, these are special tests that are being done for research and the results have no known benefit to you. Therefore, you will not receive these results. However, as a result of these tests, we expect to learn about factors that may cause breast cancer. This knowledge may be of benefit to others in the future. To minimize the risk of social stigma associated with DNA tests, the results of these tests will be kept strictly at the University of Pittsburgh. The result of these tests will not be made available to your doctor. The results of the DNA test will not bear your name, they will be identified by code numbers and will only be used for research purposes

**COSTS AND PAYMENTS:** Your biological sample or genetic material may lead, in the future, to new inventions or products. If the research investigators are able to develop new products from the use of your biological sample or genetic material, there are currently no plans to share with you any money or other rewards that may result from the development of the new product.

**SAMPLE PROVISION:**

Our knowledge of the causes of breast cancer are limited and new factors may be studied over time. We will save your samples for up to 20 years in order to try to identify genes, or factors in the blood, or factors in the urine, which will help us to understand why some women get breast cancer while others do not. We don't know exactly what will be important to test, but factors which may be studied include hormone levels in the blood and urine, infectious agents, and genes which may influence the effects of hormones, vitamin D, and dietary factors. The results of these further tests will be for research and will not be provided to you. We will use these samples for other types of studies, i.e., studies not involving breast cancer, only after asking again for your consent.

Do you accept to have your blood and urine samples stored for up to 20 years for future study?

Yes ☐ No ☐

Your refusal to sign the sample consent form does not make you ineligible to participate in this study.

Your biological samples will be stored without identifiers in University of Pittsburgh for up to 20 years or until used in approved investigations. Storage arrangements may change in response to emergency conditions or approved change in protocol. Your biological samples will be under the control of the principal investigator of this project. Your biological samples may be provided to secondary investigators without identifiers.

You may request that your biological samples be destroyed at any time and such request are to be made in writing to the principal investigator, or any of the co-investigators in Nigeria who will then pass the request to the coordinating center at Department of Epidemiology, University of Pittsburgh. Upon receipt of such a request, your samples will be pulled out from the storage using your identifier number. These samples will then be destroyed according to University of Pittsburgh protocol for disposal of biological samples.

**CONFIDENTIALITY:** Any information obtained about you in this research, including history, and laboratory data will be kept confidential. All records obtained from you in the course of this research including questionnaires and medical chart collection sheets will be transferred from the various study sites in Nigeria personally by the principal investigator and stored in a locked file cabinet in Nigeria. The records will be carried personally by the principal investigator as a hand luggage from Nigeria to the University of Pittsburgh. All records pertaining to you will be stored in a locked file cabinet at the University of Pittsburgh, and will only be accessible to the investigators listed on the first page of this informed consent form. We will give health information, for example the results of your breast examination, to your doctor or hospital at



your written request. You do understand that research records, like hospital records may be subpoenaed by court order in the United States, but this is unlikely to occur and would not affect you in any way. Your blood and urine samples will not have your name on it, but it will be identified with a code number to insure confidentiality during analysis and storage at the University of Pittsburgh. The result of the DNA testing (genotyping) will not be placed in your medical record or given to your doctor, it will be used only for research purposes. All the information that will be obtained from you will be stored in the computer database using identification numbers without your name. The questionnaires, medical chart collection sheets, data and other paper documents used in this study will be stored for five years after which they will be destroyed according to the University of Pittsburgh protocol for the disposal of research documents.

The U.S. Army Medical Research and Materiel Command are eligible to review research records as part of their responsibility to protect human subjects in research.

You consent to the publication of study results so long as the information is anonymous and/or disguised so that no one could identify you. The University Research and Conduct Office may review these research records.

**RIGHT TO WITHDRAW:** You understand that you are free to refuse to participate in this study or to withdraw at any time. Your decision will not adversely affect your care at the University of Pittsburgh, the University of Benin Teaching Hospital/Nnamdi Azikiwe University Teaching Hospital/University of Nigeria Teaching Hospital/University of Port Harcourt Teaching Hospital or your employment, nor will it cause a loss of benefits, which you might otherwise receive. You retain the right to have your blood samples and its DNA destroyed should you decide to withdraw from the research study.

**COMPENSATION FOR ILLNESS OR INJURY:** Should you be injured as a direct result of participating in this research project, you will be provided with medical care at no cost to you for that injury. You will not receive any injury compensation, only medical care. You should also understand that this is not a waiver of release of your legal rights. You should discuss this issue thoroughly with the principal investigator before you enroll in this study.

**VOLUNTARY CONSENT:** I certify that I have read the preceding, or that it has been read to me. Dr. Michael Okobia , who may be contacted at the University of Benin Teaching Hospital, 234 52 600621; or Dr. Stanley N.C. Anyanwu, Nnamdi Azikiwe University Teaching Hospital, 234 46 251912; or Dr. Emmanuel R. Ezeome, University of Nigeria Teaching Hospital, 234 42 254623; or Dr. Emmanuel E.O. Uche, University of Port Harcourt Teaching Hospital, 234 84 238923; or Dr. Clareann Bunker, University of Pittsburgh (412-624-3467) will answer any questions I have about this study. Any questions I have about my rights as a research subject will be answered by the Human Subject Protection Advocate, Institutional Review Board (IRB) Office, University of Pittsburgh (412-578-8570).

By signing this form, I agree to participate in this research study.

I, \_\_\_\_\_, consent to participate in this study.

(Please Print Name)

I understand the nature, purpose, potential risks and benefits associated with Participation, and all of my questions I have been answered.

Date \_\_\_\_\_ Signature \_\_\_\_\_

Date \_\_\_\_\_ Witnessed by \_\_\_\_\_

Permanent Address of Participant: \_\_\_\_\_

\_\_\_\_\_

#### INVESTIGATOR CERTIFICATION

I certify that I have explained to the above individual the nature and purpose, the potential benefits, and possible risks associated with participating in this research study, have answered any questions that have been raised, and have witnessed the above signature.

Date \_\_\_\_\_

\_\_\_\_\_  
**Investigator's signature**

## APPENDIX C: MAP OF NIGERIA SHOWING STUDY SITES

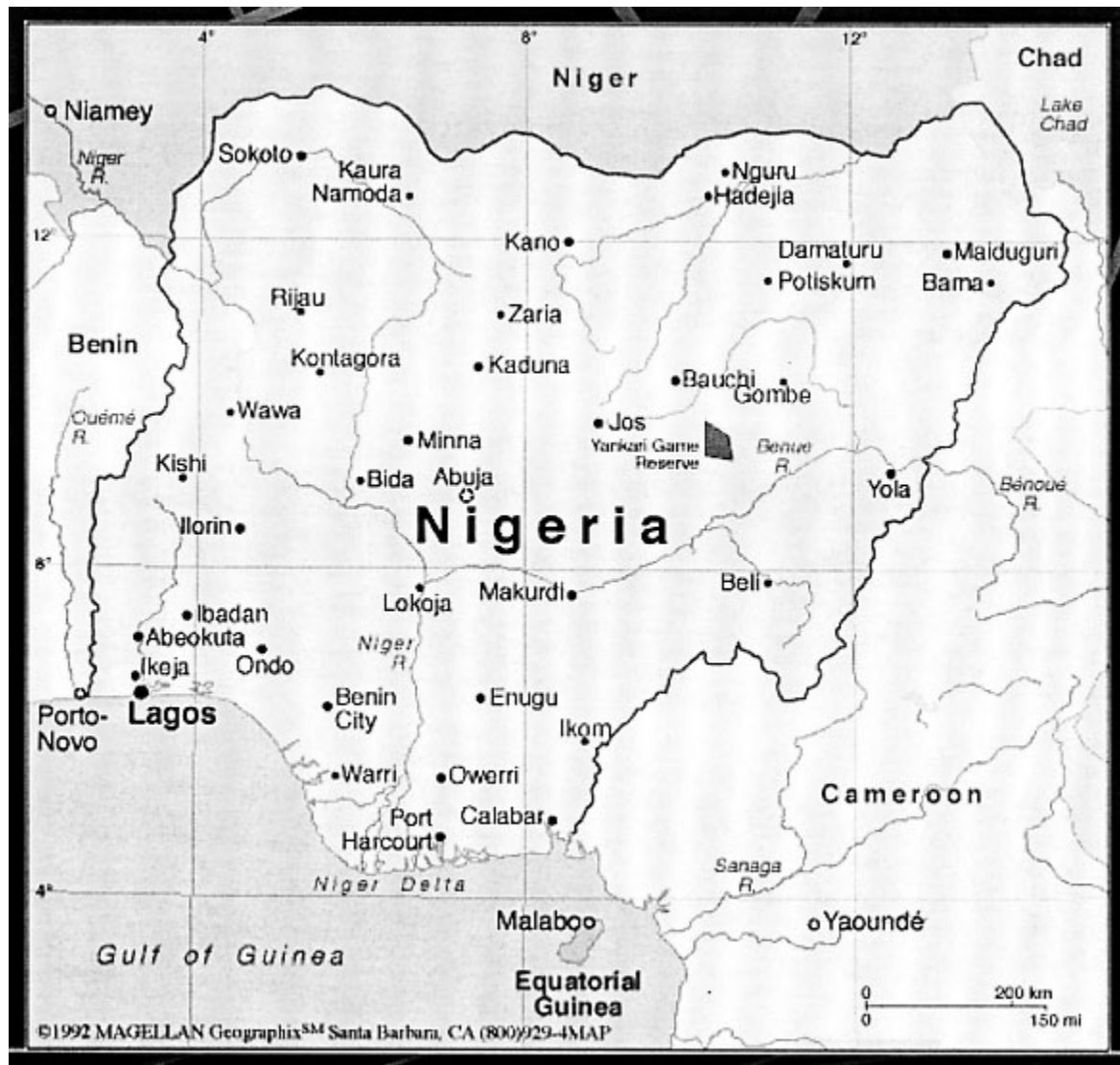


Figure 10-1: Map of Nigeria showing the Breast Cancer Study Sites

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